RADIATION QUALITY AS A DETERMINANT OF TRANSFORMED CELL PHENOTYPES

Margaret Lehane

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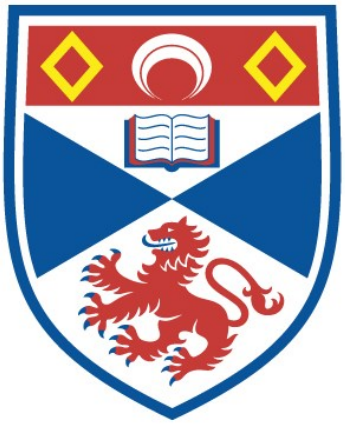

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Margaret Lehane

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To Patrick and Bridie with my love and gratitude

Abstract

Transformation is a complex multistage process *in vitro* by which benign cells gradually acquire characteristics of tumour cells. Transformed C3H10T½ cells appear *in vitro* as multilayers of cells termed foci. Two main aspects of transformation of C3H10T½ cells *in vitro* have been investigated. Firstly the quantitative assessment of the dose and dose-rate effects after irradiation with 250 kVp X-rays were examined and secondly the relationship between various properties of transformed cells *in vitro* and their tumourigenic potential *in vivo*.

The induction of transformation was found to be linear with dose for 0.25 to 5 Gy X-rays. Lowering the rate at which the X-ray dose is delivered to the C3H10T½ cells lowers the observed transformation frequency by at least a factor of two.

A variety of transformed phenotypes are observed *in vitro* and samples of these phenotypes were developed as cell lines and assessed for a number of properties. These properties were the ability to induce tumours in C3H mice and the ability to reconstruct foci *in vitro*. Other properties examined were growth *in vitro* parameters (lag time, doubling time and saturation density) as well as chromosome number and distribution. Tumour cell lines were also developed and assessed for the above properties. Transformation phenotypes induced by X-rays and alpha-particles were compared.

Differences were found between some of the properties of the X-ray and alpha-particle induced transformants. In particular higher proportions of X-ray induced transformants were tumourigenic while most of the alpha-particle induced transformants were non-tumourigenic and also tumours induced by the X-ray induced transformants appeared earlier and grew faster than the alpha-particle induced equivalent. The ability of the transformation phenotypes to reconstruct foci *in vitro* was greater for alpha-particle induced transformants (not including tumour cell lines) than for the X-ray induced transformants. The reverse was true for tumour cells where X-rays produced higher frequencies of reconstructed foci than alpha-particles. No differences were noted in *in vitro* growth parameters irrespective of transformation phenotype or radiation type apart from differences in saturation density where the transformation phenotypes (not including tumour cells) generally produced higher densities than the tumour cells for both X-rays and alpha-particles. Chromosome numbers in cells of the different transformation phenotypes (including tumour cells)

induced by both X-rays and alpha-particles showed a greater spread and a general shift of the mean and modal chromosome number to lower values than that of untransformed C3H10T½ cells. The presence of metacentric chromosomes (Robertsonian chromosomes) was not unique to the radiation induced transformation phenotypes as most of the cell lines examined showed fewer of these chromosomes than the untransformed cells. The X-ray induced transformants (including tumour cells) generally produced more Robertsonian chromosomes than the alpha-particle equivalent.

Correlation tests of the above properties with tumourigenicity of the transformed cells revealed a positive correlation of tumourigenicity with the ability to reconstruct foci. A negative correlation was noted between the ability to reconstruct foci and the mean and modal chromosome numbers.

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Chapter one

Introduction

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Ionising radiation is one of many environmental agents recognised as a potent carcinogen. It is widespread in the environment and one of the concerns for the human population is the effect of a lifetime exposure to low doses of radiation, especially with reference to the risk of cancer induction. There are many systems available to study these effects and the assay used for the purposes of this thesis is one concerned with cell transformation. *In vitro* cell transformation is the closest *in vitro* assay to carcinogenesis *in vivo*, and transformed cells gradually acquire the characteristics of tumour cells.

Radiation is a natural component of the environment. People are exposed to radiation from natural and artificial sources. The natural background of radiation exposure includes that of cosmic rays in the atmosphere, external gamma radiation from radioisotopes present in rocks and soils, internal radiation from radionuclides in the body, such as radioisotopes of potassium (^{40}K), carbon (^{14}C) and hydrogen (^{3}H), and exposure to radon and its decay products (Sumner 1987). Artificial sources of radiation include the fallout from nuclear weapons testing, radioactive waste, for example, from radioisotope users and nuclear establishments, and occupational exposure. Medical exposure to radiation can be due to diagnostic X-rays, nuclear medicine and radiotherapy treatment for cancer. Over seventy percent of the lifetime exposure of individuals to radiation is due to natural sources of radiation and approximately twenty percent of exposure is from medical sources while the remaining exposure is comprised of occupational and other exposures to artificial sources of radiation (data summarised in UNSCEAR 1993).

One of the major concerns arising from exposure to radiation is that of the risk of cancer induction. Cancers induced by radiation are morphologically indistinguishable from those induced by other agents although differences on the molecular level are being reported (for example, Vähäkangas *et al.* 1992, Taylor *et al.* 1994, Hillebrandt *et al.* 1996). The latency period for many cancers makes it difficult to definitively identify the causative agent. Much of the evidence for the carcinogenic effects of radiation has evolved from epidemiological studies of people exposed to

higher than average doses of radiation. Some of these studies indicate an excess incidence of cancer in exposed groups over the natural or expected incidence. The largest group of exposed individuals are the Japanese atomic bomb survivors from Hiroshima and Nagasaki. Other groups examined are ankylosing spondylitics who received radiotherapy to relieve pain associated with the disease, women treated for carcinoma of the cervix, children exposed *in utero* to diagnostic X-rays before 1958, patients irradiated for *tinea capitis*, Hodgkins disease, ovarian cancer and leukaemia, as well as the residents of areas in India, Brazil, Colorado and China with high natural background levels of radiation (ICRP 1990). Groups of workers exposed occupationally to high levels of radiation are underground miners, radium dial painters and chemists (Fabrikant 1991). These groups collectively form the basis for the epidemiology studies used to assess risk of cancer induction by exposure to radiation.

Most of the data available on the health effects of exposure to ionising radiation relate to high doses and high dose-rates. Information about low doses and low dose-rates is mostly extrapolated back from the high dose data, most often derived from the epidemiology studies of people exposed to low-LET radiation (data summarised in UNSCEAR 1993). The LET (linear energy transfer) at a point on the track of an ionising particle represents the energy absorbed by the medium per unit length of track and is usually expressed in keV/μm (Tubiana *et al.* 1990). To determine the risk of radiation-induced cancer at low doses over extended periods of time, it is assumed that the dose-response relationship is linear, that is, the derived risk is proportional to the dose. However at low doses and dose-rates, there is evidence of a reduced effectiveness, especially for low-LET radiations (data summarised in UNSCEAR 1993). Because of this, a Dose and Dose-Rate Effectiveness Factor (DDREF) is applied to the risk estimates derived from the high doses and dose-rates. The values of DDREF vary, from 2 (ICRP 1977, 1990) to 2-10 (UNSCEAR 1993, BEIR 1990). Most radiation received in a lifetime is likely to be chronic low dose exposure from environmental, occupational, or medical sources. The uncertainties inherent in the radiation risk estimates at low doses and dose-rates are quite considerable when one combines uncertainties on data at high doses and dose-rates

with the uncertainties in the calculation of DDREF.

One of the principal objectives of *in vitro* cell biology studies is to determine the dose - response relationship at low doses of ionising radiation, obtain more precise data on the values of DDREF by assessing dose-rate effects at low doses, and assess relative effects of different types of radiation. Previous cell transformation studies carried out in this laboratory examined the effect of protracting the dose for neutrons and alpha-particles on the transformation frequency (Mill *et al.* 1994, Hall *et al.* 1991). Data on the effect of protracted X-ray doses will be presented in this thesis (see chapter four).

1.1. Transformation

The term transformation may have several meanings in biology and in the context of this thesis it is described as the transition of a pre-neoplastic cell to the neoplastic state. The term is applied to *in vitro* studies and is distinct from the term carcinogenesis which applies *in vivo*, although they may have some common features. The transformation of a cell involves change(s) which manifest in numerous ways *in vitro*. Some of these changes include those allowing the growth of cells as tumours in nude mice, those leading to focus formation *in vitro* and growth of cells in soft agar. The many changes associated with the transformation of cells have been documented for animal cells and human fibroblasts (Borek and Ong, 1989). Transformed fibroblasts (hamster and mouse cell studies) *in vitro* become pleomorphic with refractile criss-cross orientation and an irregular growth pattern when transformed. They also show increased saturation density, growth in multilayers and loss of density-dependent inhibition of growth (Borek 1985).

Cell transformation is the closest *in vitro* assay to carcinogenesis *in vivo*. Carcinogenesis and transformation are both regarded as multistage processes although transformation *in vitro* is studied in cells which have already undergone some of the possible steps, for example, immortalisation. There are several reasons for assuming the validity of a multistage model, which was derived in an attempt to explain the age-incidence curves for cancer induction in humans (Woodruff 1990). Abnormal cells

(histologically, karyotypically or functionally) are commonly found close to cancer sites *in vivo* or at sites where cancer subsequently develops, for example in some cases of colorectal cancer. Many oncogenes cannot transform normal cells when acting alone but will do so in co-operation with another oncogene or an immortalising agent (Woodruff 1990). Some studies indicate that radiation transformation is not due to one single oncogene activation but rather a combined effort of a number of genes (Shuin *et al.* 1986, Borek *et al.* 1987). Although the sequence of events in transformation and carcinogenesis are considered to be initiation, promotion and progression (Pitot *et al.* 1991) these terms have different meanings when one refers to transformation versus carcinogenesis. Cells examined in transformation studies are already altered to some extent, for example by immortalisation, thus the term initiation may implicate a change in immortal cells in transformation and a change leading to immortalisation in carcinogenesis. Initiation of transformation or carcinogenesis involves the exposure of cells to a carcinogen and is thought to reflect a permanent, irreversible change in the cell. The promotion stage is considered to be reversible and modified by the environment. It involves increasing the proliferation rate of the cells and thereby increasing the risk of cancer development. Progression may be used to describe the final stages of carcinogenesis or transformation which may ultimately lead to tumour development *in vivo* in the case of carcinogenesis or the formation of foci in tissue culture experiments in the case of transformation (Pitot *et al.* 1991). One of the best examples of the multistage process of carcinogenesis is colorectal cancer (Fearon and Vogelstein 1990, Williams *et al.* 1990). Clinical and histopathological data suggest that most if not all malignant colorectal tumours arise from pre-existing benign tumours and that colorectal tumours appear to arise as a result of mutational activation of oncogenes coupled with mutational inactivation of tumour suppressor genes (Fearon and Vogelstein 1990). The total accumulation of changes in at least four to five genes is important for malignancy, not necessarily the order in which those changes occur (Fearon and Vogelstein 1990).

The ability of radiation to transform cells *in vitro* has been recorded in animal cells (for example, Borek 1979, Miller *et al.* 1979, Gould *et al.* 1991, Watanabe *et al.* 1989, Terasima and Yasukama 1989, Hall *et al.* 1989, Servomma and Rytömaa 1990)

and in human cells (for example, Barendsen 1989, Redpath *et al.* 1989, Guolian *et al.* 1989). Radiation induced transformation *in vitro*, studied in C3H10T½ cells is thought to be initiated by a high frequency event occurring in the irradiated cells (for example, a change in gene expression) with a later event required for complete transformation (Kennedy 1989). This thesis examines the differences, in a particular transformation assay the C3H10T½ cell assay (Reznikoff *et al.* 1973) described below, between unirradiated non-transformed parent cells and cells transformed by low- and high-LET radiations.

Transformation assays

Experimental studies of carcinogenesis using experimental animals have yielded important quantitative data, however they have their limitations in studies concerned with the effects of low doses of carcinogens where large numbers of animals are required, and in the investigation of cellular and molecular events in carcinogenesis induced by radiation. The development of cell culture systems has made it possible to study the effects of radiation under defined conditions, without the input of the complex metabolism of a host animal. The use of cell cultures also reduces the cost, time and ethics problems associated with animal experiments.

Animal cell systems in use include the mouse C3H10T½ system (Reznikoff *et al.* 1973), the 3T3 mouse cell system (Daya-Grosjean *et al.* 1989), hamster embryo cells (Bols *et al.* 1989, Watanabe *et al.* 1989, Hall and Hei, 1985), rat tracheal epithelial cell lines (Nettesheim *et al.* 1989, Thomassen 1989) and the rat granuloma pouch assay (Mohn *et al.* 1989).

A variety of human cell lines have been developed using a number of immortalising agents. Examples include epithelial cells immortalised with the human papilloma virus (DiPaolo *et al.* 1989), skin epithelium, urothelial cells and fibroblasts all immortalised with SV40 DNA (Fusenig *et al.* 1989, Reznikoff *et al.* 1989, Hoffschir *et al.* 1989), skin fibroblasts immortalised by fusion with HeLa cells (Redpath *et al.* 1989), and thyroid cells immortalised by SV40 DNA transfection (Lemoine *et al.* 1989). Other agents have also been shown to be capable of immortalising cells, for example, fibroblasts treated with Cobalt-60 gamma rays

(Namba *et al.* 1989, Borek and Ong, 1989), or human colorectal cells immortalised through prolonged culture *in vitro* (Paraskeva *et al.* 1984).

Immortalised cell lines possess an unlimited life span and arise from cell populations which originated as primary tissue cultures (Adams 1988). The advantage of immortal cell lines is the ability to maintain stocks of cells at a particular passage and the resultant improved reproducibility of experimental data. Immortalised cell lines provide a homogeneous population of cells which can be derived from single parent cells. The availability of a large stock of cells of defined characteristics allows improved reproducibility of results and studies both within a laboratory and between different laboratories (Kakunaga 1985). However these cells have also undergone some of the changes, such as immortalisation, associated with transformation and carcinogenesis. Primary tissue cultures are also used in radiobiology studies. Primary cells are freshly derived from animal or human tissue, are direct descendants of the cells *in vivo*, consist of diploid cells and have a finite life span in culture. They are limited in their use by the constant need for a source of tissue and results are less reproducible as a result but probably of greater relevance than some immortalised cell lines as the cells are closer to the *in vivo* state than immortalised cell lines and have undergone less changes.

The C3H10T½ mouse embryo cell system

The C3H10T½ cell line originated from C3H mouse embryo cells, which became immortalised with passaging in culture. C3H10T½ cells are highly sensitive to inhibition of cell division once confluence is reached when they form a continuous monolayer of cells (Reznikoff *et al.* 1973). The cell line can be easily manipulated, has a low saturation density and a low spontaneous transformation frequency.

C3H10T½ cells were described as fibroblast-like with long cytoplasmic processes. Cells grow in flat even monolayers with an epithelial-like appearance in confluent cultures. Cells are hypertetraploid and non-tumourigenic in C3H mice (Reznikoff *et al.* 1973). The cell line has been widely used as a transformation assay for both chemical and radiation effects (for example, Balcer-Kubiczek *et al.* 1994, Hall *et al.* 1991, Kennedy and Little, 1984, Hill *et al.* 1985, Krolewski and Little 1994,

Smith *et al.* 1993).

Foci produced when C3H10T½ cells were exposed to a carcinogen were classified into three types by Reznikoff *et al.* (1973). Type I foci (see figure six in section 5.1.), composed of tightly packed cells were not scored as malignantly transformed, since isolated foci failed to produce tumours in C3H mice. Type II foci showed considerable piling-up of cells into virtually opaque multilayers, in which cells were only moderately polar and criss-crossing of the cells was not pronounced. The third category of focus (type III) consisted of highly polar, fibroblastic, multilayered criss-crossing arrays of densely stained cells (see figure one in section 5.1.). Types II and III are classified as malignantly transformed, since fifty percent of type II and eighty five percent of type III produced tumours in C3H mice (Reznikoff *et al.* 1973).

The standard C3H10T½ transformation assay as devised by Reznikoff *et al.* (1973) involves exposure of the cells to the carcinogen, followed by subculture of the exposed cells which are then allowed to grow to confluence. Confluence is maintained for three to four weeks and cultures are then stained and examined for focus formation.

A number of factors, other than the radiation or chemical treatment affect the number of foci observed and thus the transformation frequency calculated from the C3H10T½ assay. These include the quality of the foetal calf serum used in the growth medium, the cell seeding density immediately after irradiation, the cell cycle phase at the time of irradiation and the presence of some anti-microbial agents, promoters or inhibitors of transformation. The observed transformation frequency decreases as the initial cell seeding density increases above a certain density (Reznikoff *et al.* 1973, Terzaghi and Little 1976, Haber *et al.* 1977, 1978, Bettega *et al.* 1989). The percentage of foetal calf serum used in the growth medium for the transformation assay can reduce the observed transformation frequency induced by carcinogens. This is reversed by reducing the level of serum in the growth medium for a sufficient period of time, for example once confluence is reached (Bertram 1977). It is therefore important to ensure that the serum used in the assay will support good growth of both transformed and untransformed cells.

The presence of a promoter such as 12-o-tetradecanoylphorbol-13-acetate

(TPA) or active oxygen species enhances the transformation of C3H10T½ cells by radiation and chemicals (Kennedy and Little 1978, Little *et al.* 1979, Han and Elkind 1982, Zimmerman and Cerutti 1984). The activation of protein kinase C by TPA appears to be an important step in its action on transformation of C3H10T½ cells (Hei *et al.* 1994). The presence of protease inhibitors, retinoids or ascorbic acid can reduce the number of transformed foci observed and although the exact mechanisms are unknown those studied all seem to act on the promotion stage of transformation (by chemicals) by increasing the time needed for expression of transformation (Kuroki and Drevon 1979, Merriman and Bertram 1979, Benedict *et al.* 1980).

Early protocols for the C3H10T½ transformation assay involved the use of the antibiotic penicillin in the growth medium. This has been superseded, for the most part by gentamicin, since penicillin was found to reduce the number of transformants observed after treatment with chemicals or radiation (Bertram 1979).

The transformation frequency of the C3H10T½ cell line also depends on the cell-cycle phase the cells were in when irradiated. Cells in various phases of the cell cycle vary in their sensitivity to radiation for both cell killing and transformation (Miller *et al.* 1992, Cao *et al.* 1993). Mitosis was the most sensitive phase for cell killing and late S and G_1 phases the most resistant phases when cells were treated with X-rays. Cells were most resistant to transformation by X-rays when irradiated in the S phase and most sensitive in late S / early G_2 phases (Miller *et al.* 1992, Cao *et al.* 1993).

A number of theories have been postulated to account for the process of transformation, based on work using the C3H10T½ system. Haber *et al.* (1977) and Bertram (1977) proposed transformation occurred as a result of a single event in treated cells, and the expression of transformation was suppressed by contact with normal cells. This explained the reduced transformation frequency observed when treated cells were initially seeded at high cell densities. These results are consistent with the two event model proposed by Kennedy and Little (1980, 1984) who proposed that the first (initiating) event is relatively frequent, involving a large number of treated cells, and the second event is rare, involving the descendent(s) of the treated cells before the cells reach confluence. They proposed the second event behaved like

a spontaneous mutation with a small but constant probability of occurrence every time an initiated cell divided. This constant probability per cell per generation indicated that the yield of transformed foci would rely on the number of cell divisions by initiated cells in the treated population. Fernandez *et al.* (1980) proposed a third event involving the reversion of a fraction of the "initiated" cells to the untransformed state before the "second" event occurred. A later proposal was that the second event described by Kennedy and Little may be comprised of a number of events (Kennedy 1989, Little 1985).

Backer *et al.* (1982) reported that the commitment of C3H10T½ cells to transform occurred within two days of exposure to the carcinogen. Mordan *et al.* (1983) determined from their studies on the transformation assay that the total number of initiated cells present at confluence did not determine the number of transformed foci but rather the distribution of these cells in colonies of appropriate size was directly related to the expression of the transformed phenotype. Thus the transformation frequency was independent of the total number of cell generations.

Spontaneous focus formation has been observed to be independent of the initial surviving cell densities but related to the number of cell divisions between the time of cell seeding and the suppression of the proliferation of the untransformed cells (Grisham *et al.* 1988). The spontaneous frequency was consistent with that of a single gene locus mutation.

Properties of transformed C3H10T½ cells

There are several phenotypic properties characteristic of transformed C3H10T½ cells *in vitro*, these include changes in cell morphology and cell organisation in colonies and foci, increased saturation density, ability to grow in reduced serum levels and in agar. Transformed cells can be distinguished by scanning electron microscopy from untransformed cells by the formation of foci (random piling of cells on top of each other), pleomorphism in cell size and shape, and cell surface complexity, but the cells were only distinguishable on a population basis (Narayan *et al.* 1984). Transformation by radiation of C3H10T½ cells may produce alterations in the cell membrane, seen by variations in the order of the structural components of the

membrane at low temperatures, most likely due to changes in its glycoprotein content (Grossi *et al.* 1992). C3H10T½ parent cells have a reduced calcium requirement for initiation of DNA synthesis compared to primary fibroblasts but the calcium requirement of transformed C3H10T½ is reduced even further (Kakunaga 1985).

Smith *et al.* (1993) reported in their study of chemically transformed C3H10T½ cells that the greatest difference between tumourigenic and non-tumourigenic focus-derived cell lines was in focus reconstruction studies where the ability of the transformed cells to reproduce foci on confluent monolayers of untransformed C3H10T½ cells was examined. For the other transformation parameters (except doubling time), the results from all clones differed significantly with those of the untransformed C3H10T½ cells tested. The morphology did not correlate well with the tumourigenicity of the foci and the results of the focus reconstruction studies correlated better with tumourigenicity than those of the anchorage independence assay.

1.2. Transformation and the dose-rate effect

As stated previously, the major concerns regarding the biological effects of radiation and the risk of cancer induction are the effects at low doses and low dose-rates. *In vitro* cell systems can be used to examine the effect of prolonging the radiation exposure time. Several studies using the C3H10T½ cell transformation assay have been carried out to study the effect of protracting the exposure to high- and low-LET radiations.

High-LET dose-rate effects

The results of dose-rate and dose-fractionation experiments are controversial. Previous studies in this laboratory using the C3H10T½ cell transformation assay examined the effect of lowering the dose-rate for neutrons (2.5 MeV) and alpha-particles (120 keV/μm). No dose-rate effect was observed for alpha-particles (there was some indication of a reduction in the transformation frequency with prolonged exposure time for cycling cells and a smaller reduction for plateau phase cells but neither was significant). However an increase in the transformation frequency by a

factor of 1.3 with dose-rate (0.6 mGy/min and 20 mGy/min) was observed for a dose of 0.2 Gy of 2.5 MeV neutrons (Hall *et al.* 1991).

Han and Elkind (1979) fractionated 3.78 Gy of neutrons and found no change in the cell survival of C3H10T½ cells, but a reduced transformation frequency over a twenty four hour period by a maximum factor of 1.7. Hill *et al.* (1985) reported a significant enhancement (approximately a factor of eight) of neutron-induced transformation with protraction of the dose. The results supported the hypothesis that the repair of the damage induced by protracted exposures of neutrons may be "error prone" and/or may facilitate the expression of subeffective transformation damage (Hill *et al.* 1985).

Data of Miller *et al.* (1988, 1991) qualitatively agreed with Hill's data but Miller reported that the enhancement was by a maximum factor of two, and depended on the energy of the neutrons; no dose-rate effect was observed with 13 MeV (LET 130 keV/μm) neutrons and the greatest effect was with 5.9 MeV (LET 75 keV/μm) neutrons. This neutron energy dependent effect was supported by Yasukawa *et al.* (1987) who reported an enhanced transformation frequency with dose fractionation but only with 2 MeV neutrons produced in a Van de Graaff generator, not with 13 MeV cyclotron-produced neutrons. It was also reported that the fractionated responses were intermediate between the continuous low dose-rate and the high dose exposures (Miller *et al.* 1991). However Balcer-Kubiczek *et al.* (1988, 1994), Saran *et al.* (1994), and Ullrich (1986) failed to observe a dose-rate effect with neutrons.

Hieber *et al.* (1987) found no enhancement of transformation frequency with protraction of the alpha-particle (LET = 147 keV/μm) exposure in an analogous experiment to that done by Hill *et al.* (1984) which found a significant enhancement with neutron dose fractionation. Bettega *et al.* (1989) initially reported no inverse dose-rate effect with alpha-particles (LET = 101 keV/μm) but later (1992) reported an inverse dose-rate effect of 1.4 with alpha-particles (LET = 101 keV/μm) using two separate fractionation schemes. These results corresponded with data by Miller *et al.* (1991) in showing an inverse dose-rate effect of two.

Further data of Miller *et al.* (1993) using single and fractionated doses of charged particles of defined LET showed a range of LET values (40 -120 keV/μm)

within which an enhanced transformation frequency with dose protraction is observed. It is suggested that the inverse dose-rate effect disappears at high-LET because of the reduction in the number of cells being hit and at low-LET because most of the dose is deposited at low specific energies, insufficient to produce a saturation effect (Brenner *et al.* 1993). At even lower LET (X-rays and gamma rays) damage repair yields a sparing effect. This limited LET range for inverse dose-rate effects complicates the practical applications in radiological protection. One example of such an application would be the assessment of the risk of lung cancer induction due to domestic exposure to radon gas, by extrapolation from data on underground miners exposed to much higher levels of alpha-particles. Most exposures to radon daughter alpha-particles are at LETs of 150-200 keV/μm. At low doses, such as domestic exposures, no dose-rate effect would be expected as multiple hits of the nuclei would be unlikely. However at higher doses such as those received by some miners, the analysis of the data may be complicated by inverse dose-rate effects, which may cause the radiation risk estimations for environmental exposures to be overestimated (Brenner *et al.* 1993).

Elkind (1991) reported possible technical reasons for the differences between various authors on the presence or absence of a dose-rate effect for high-LET radiations. One reason was the potential loss or incomplete irradiation of the transformation sensitive mitotic cells. These cells are spherical in culture and more loosely attached to the culture dish. The author proposed that the rounded mitotic cells were insufficiently irradiated due to the limited range of alpha-particles, for example, to explain the absence of an inverse dose-rate effect reported by Hieber *et al.* (1987). Also, in experiments which involve transportation of cells from an irradiation site to the laboratory, the transportation and subsequent processing of the samples may result in loss of the loosely attached mitotic cells.

Low-LET dose-rate effects

Data on cell transformation with low-LET radiations indicate a reduced effect at low dose-rates compared with acute doses. Studies show a reduction of the transformation frequency with protraction of the dose for gamma rays and for doses

of X-rays greater than 1 Gy (Metalli *et al.* 1969 (tumour induction in mice), Terzaghi and Little 1976, Miller *et al.* 1978, 1979, Han and Elkind 1979, Wells and Bedford 1983, Watanabe *et al.* 1984 (golden hamster embryo cells), Hill *et al.* 1984, 1987, Yang *et al.* 1986, Balcer-Kubiczek *et al.* 1987).

Balcer-Kubiczek *et al.* (1987) concluded that the time for repair of the transforming damage during the irradiation was the determining factor of the dose-response relationship for transformation at reduced dose-rates. Studies indicated that although subeffective preneoplastic transformation damage can be repaired during a protracted exposure of X-rays or gamma rays, some damage which is operationally similar after both high and low dose-rates persists for at least twenty four hours (most of the potentially transforming damage is repaired within six hours of irradiation (Terasima *et al.* 1985)) because it can then be made evident by the addition of a promoting agent such as TPA (Hill *et al.* 1984, 1987, Balcer-Kubiczek *et al.* 1989).

The available data for doses less than 1 Gy X-rays is not consistent. Miller *et al.* (1978, 1979) fractionated doses of X-rays and found that the dose-response curve for transformation induction crossed at about 1.5 Gy X-rays. Below this dose the fractionated dose was a more effective inducer of transformation, while above this dose it was less effective than the dose given as a single exposure. Enhanced transformation frequencies at lower doses were also found in golden hamster embryo cells (Borek *et al.* 1974), but not in plateau phase C3H10T½ cells (Terasima *et al.* 1985). Decreases in mutation induction in Chinese hamster V79-S cells have been reported for low dose-rates compared to high dose-rates of ^{60}Co gamma rays (Crompton *et al.* 1990). However further reductions in dose-rate resulted in a reverse dose-rate effect and an increase in the frequency of mutants was observed (Crompton *et al.* 1990). Data on the X-ray dose-rate effects on the transformation frequency of C3H10T½ cells will be presented in this thesis (see chapter four).

1.3. Cytogenetics

There is strong circumstantial evidence that damage to DNA (deoxyribonucleic acid) which carries the genetic information in chromosomes in the cell nucleus is

considered to be the main target for the biological effects of ionising radiation, including cell killing, mutation and carcinogenesis (for example, UNSCEAR 1993, Hall 1994). The damage may be lethal, sublethal or potentially lethal in nature. Lethal damage is irreversible and irreparable leading to cell death, while sublethal damage can be repaired under normal circumstances unless the irradiated cells receive additional damage. Potentially lethal damage represents the component of radiation damage that can be modified by post - irradiation environmental conditions (Hall 1994).

DNA damage caused by radiation may be direct through the action of the radiation on the DNA molecule or indirect through the production of active molecular oxygen species, water radicals or thymine glycols which interact with the DNA molecules (Leadon 1990). This DNA damage may take the form of base damage, single- or double- strand breaks and multiple damage sites in the DNA (Tubiana *et al.* 1990, Ward 1995). The number of single- strand breaks is considered linear with dose while the relationship between radiation dose and the number of double- strand breaks is more complex although also linear under some measurement conditions (for example Blöcher 1982, Frankenberg *et al.* 1984, Okayasu and Iliakis 1989). The contribution of base damage to the cellular effects of radiation is unlikely to be significant at the level produced by most doses of ionising radiation while the interaction of lesions close in space and time is regarded as highly significant (Ward 1995). Local multiple damage sites (LMDS) or clustered damage arises when the energy of the radiation is deposited in a sufficiently small area to cause multiple damage on a local site of the DNA (Ward 1995). Since high-LET (linear energy transfer) radiation deposits a greater energy per unit distance than low-LET, multiple damage sites are more likely to occur following exposure to high-LET radiation (Ward 1994). The measurements of the initial yields of single- and double- strand breaks as a function of LET indicate only a moderate increase by a factor of two to three even at high-LETs which show significant differences for other parameters such as cell killing as a function of LET (Brenner and Ward 1992, Ward 1994). Two hypotheses which have been proposed to explain the observed increase in cell killing with increasing LET are firstly that as the LET increases the double- strand breaks are

formed closer together making interaction of the breaks to form chromosomal exchanges more probable (Kellerer and Rossi 1978, Brenner 1990) and secondly that as the LET increases the complexity of the double- strand breaks increases making them less capable of being repaired (Ward 1981).

The cellular response to DNA damage is to attempt to repair the damage. Misrepair of the damage leads to aberrations in the chromosomes. These aberrations are of two main classes, chromosome and chromatid aberrations. Chromosome aberrations result if the cells are irradiated early in interphase before the chromosome material has been copied. The radiation induced break occurs in a single strand of chromatin which is then duplicated during the synthesis phase of the cell cycle leading to a chromosome aberration visible at the next mitosis because identical breaks appear in the corresponding points of a pair of chromatin strands. Chromatid aberrations arise when the cells are irradiated after the DNA material has doubled and the chromosomes consist of two strands of chromatin. The radiation induced break may occur on one or both of the sister chromatids (Hall 1994). Aberrations are generally regarded as stable (balanced translocations) or unstable (dicentrics, rings and fragments) (see figure one). It is generally assumed that translocations and dicentrics are formed in equal proportions following exposure to X-rays. The advent of more specific techniques such as fluorescence *in situ* hybridisation (FISH) provides data that indicate the frequency of translocations is about twice that of dicentrics for a given dose of X-rays (data reviewed in Natarajan *et al.* 1994), although more recent data reverts to the historical assumption of equal proportions of dicentrics and translocations produced after irradiation (Fernandez *et al.* 1995, Hande *et al.* 1996). A higher incidence of complex chromosomal exchanges involving three or more breaks in two or more chromosomes has also been reported than previously suspected at least for the higher doses (> 2 Gy) of low-LET radiation (Simpson and Savage 1994).

These different types of aberrations may result in the activation of proto - oncogenes or the inactivation of tumour suppressor genes possibly causing the initiation of carcinogenesis (UNSCEAR 1993). In some human neoplasia the early phases of development are often associated with consistent chromosomal rearrangements, for example the translocation chromosome (Philadelphia chromosome)

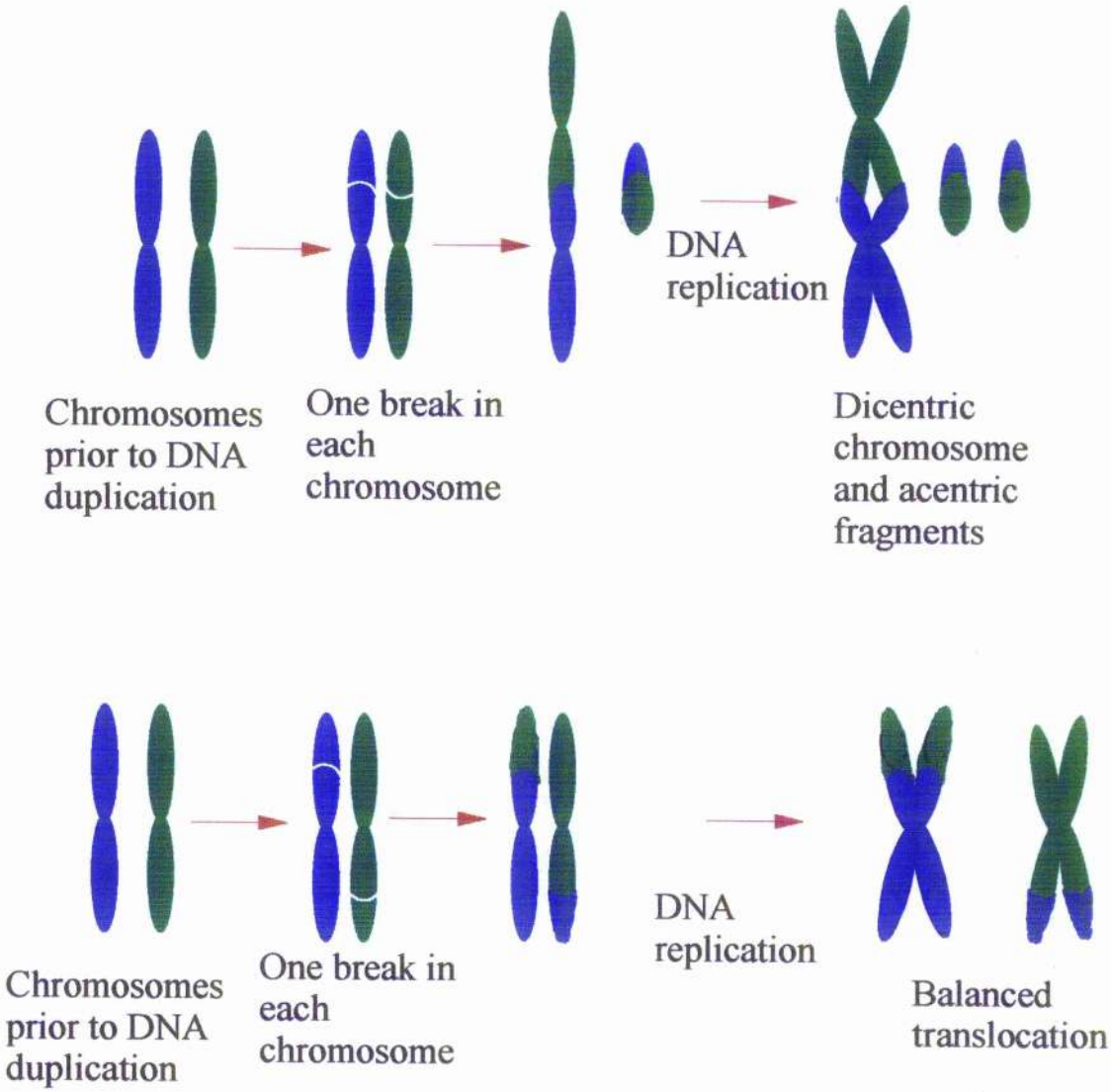

Figure 1.1. Chromosome aberrations. Figure shows the formation of a dicentric chromosome and acentric fragments and the formation of balanced translocations. These are the most common aberrations observed after radiation exposure.

formed between chromosomes 9 and 22 in chronic myeloid leukaemia (results in fusion of *bcr* and c-*abl* genes), between chromosomes 14 and 18 in follicular lymphoma and between chromosomes 8 and 14 in acute lymphocytic leukaemia (UNSCEAR 1993, Rabbitts 1994). Many of these translocations activate proto-oncogenes, for example c-*myc* activation in Burkitt's lymphoma, resulting in the production of fusion proteins or otherwise affect the transcription factors which influence other genes elsewhere in the genome (Rabbitts 1994).

There is considerable evidence that ionising radiation triggers genomic instability and that radiation - induced double- strand breaks are an important trigger of this instability (for example, Kronenberg 1994, Kadhim *et al.* 1992, Sabatier *et al.* 1992, 1994). Instability has been observed as non-clonal aberrations appearing several cell generations after the initial radiation exposure, usually observed after high-LET radiation exposure (Kadhim *et al.* 1992, Gorgojo and Little 1989, Sabatier *et al.* 1992, 1994, Marder and Morgan 1993). The process of genomic instability may be seen as a balancing act where the affected cells evolve at an increased rate either leading to the accumulation of lethal mutations and thus cell death or alternatively to the increased chance of attaining mutations leading to a growth advantage (Kronenberg 1994). The instability could greatly increase the frequency of spontaneous and induced genetic changes with some data supporting the loss of one of the cell cycle control checkpoints actually leading to genomic instability and thus inappropriate survival of genetically damaged cells and the development of the cells to malignancy (Hartwell and Kastan 1994). A striking example of genomic instability correlated with human cancer predisposition is that of hereditary non - polyposis colorectal cancer (HNPCC) where the tumours show widespread alterations in short DNA repeat sequences although a possible role of these repeat sequences in genomic instability has not been elucidated (UNSCEAR 1993).

Cytogenetic studies of C3H10T½ cells

Untransformed C3H10T½ cells are hypertetraploid with a small proportion of cells in the octaploid range (Reznikoff *et al.* 1973). A minute marker was found in ninety three percent of the cells. The modal chromosome number for transformed cells

remains near tetraploid, with a greater distribution of chromosome number around the mode. The number of octaploid cells and the number of cells with more than one minute chromosome were increased in the transformed cells examined. Two of the four clones examined had large marker chromosomes (one metacentric, one acrocentric) in a large number of cells (Reznikoff *et al.* 1973). The presence of double minute chromosomes and homogenously staining regions are usually associated with amplified DNA sequences and not generally found in irradiated cells, but recent studies have reported them in radiation transformed focus cell lines (Privitera *et al.* 1990).

Crompton *et al.* (1994) analysed the DNA content of X-ray transformed C3H10T½ cells and most of the clones showed multiple ploidy states, even in clones originally isolated from single cells. A reduction in the number of cells showing the higher ploidy states often occurred with cell growth and passage, resulting in the reversion of the population to the hypertetraploid state of the parent cells (Crompton *et al.* 1994). These data do not concur with the findings of Smith *et al.* (1993) where eighty percent of the transformed C3H10T½ clones (chemically transformed) had lost considerable amounts of DNA even in the passages in culture required to establish the cell lines. Crompton *et al.* (1994) deemed these results to highlight differences between transformation by chemicals compared to X-rays.

Genomic rearrangements with loss of a specific DNA region have also been observed in X-ray transformed C3H10T½ cells (Paquette and Little 1992). X-rays produce more exchanges than deletions in C3H10T½ cells while alpha-particles produced more deletions than exchanges in all phases of the cell cycle (Durante *et al.* 1992, 1994). The relationship between chromosome aberration induction in cells irradiated in plateau phase is linear with dose for alpha-particles and proportional to the dose squared for X-rays (Nagasawa *et al.* 1990, Durante *et al.* 1994). Both types of radiation produced a significant increase in the number of Robertsonian translocations when the cells were irradiated in the G_1 or S phase but not in G_2. Both Robertsonian translocations and sister chromatid exchanges occur at a relatively high frequency, probably related to mitotic crossing over rather than double strand breaks. The untransformed C3H10T½ cells had a high level of spontaneous Robertsonian

translocations (Durante *et al.* 1994).

Robertsonian fusions were first described by W.R.B Robertson in 1916 who concluded from observations that a metacentric chromosome in one species may correspond to two acrocentrics in another that during evolution metacentrics may arise by fusion of acrocentrics (Robertson 1916). Whole chromosome arm fusions are called Robertsonian translocations in his honour. They are the most common structural chromosome abnormality in humans, one in a thousand in the general population (Wolff and Schwartz 1992), can occur spontaneously and are not thought to be specifically induced by ionising radiation.

The cell lines isolated from foci in this thesis work (X-ray and alpha-particle induced) were examined for changes in the tetraploid state of the cells and for the presence or absence of Robertsonian translocations and the results are presented in section 5.5.

Oncogenes

Oncogenes are a set of genes which can cause cells to become malignant if their expression is altered by mutation or overexpression. Much work has been done to try and identify oncogenes which may be involved in the transformation of C3H10T½ cells. X-ray transformed cell lines examined for oncogene mutations showed no gross rearrangements or amplifications of v-Ha-*ras*, v-Ki-*ras*, N-*ras*, v-*myc*, v-*raf*, v-*src*, v-*fes*, v-*abl*, v-*mos*, v-*erb*-A, v-*erb*-B, v-*myb*, *neu*, *trk*, *fms*, v-*fos* or v-*sis* oncogenes (Shuin *et al.* 1986, Borek *et al.* 1987, Krolewski and Little 1989, 1994, Privitera *et al.* 1990, Thomas and Guernsey 1991). An enhanced level of c-*myc* protein (without structural change in the gene), decreased level of c-*fos*, structural changes in the p53 gene, enhanced mRNA levels of *raf* and mutated c-K-*ras* genes have been reported in some transformed C3H10T½ cell lines (Shuin *et al.* 1986, Chen and Herschman 1988, Thomas and Guernsey 1991, Leuthauser *et al.* 1992, Smith *et al.* 1993, Krolewski and Little 1993, 1994). It is not known whether the mutation in the *ras* gene is involved in transformation since the mutated allele appears not to be expressed and was not enhanced in all the transformed C3H10T½ cell lines examined (Thomas and Guernsey 1991). Transfection of C3H10T½ cells with an exogenous c-

myc oncogene greatly enhances the sensitivity to transformation (Sorrentino *et al.* 1987), thus *c-myc* appears to act as an accomplice in transformation, possibly by enhancing the cells' response to growth factors. An overexpression of the mutant p53 protein in a number of chemically transformed C3H10T½ cell lines correlated well with the tumourigenicity of these cells (Coleman *et al.* 1994). The results to date indicate that X-rays appear to activate as yet unidentified oncogenes. However the complex effect of the radiation on epigenetic mechanisms, genome stability, mitotic recombination, RNA levels, protein levels and other factors which affect proliferating cells probably also have a significant role to play in transformation.

1.4. Objectives

The objective of this thesis was to examine a wide range of tumourigenic and non - tumourigenic C3H10T½ foci, induced by high- and low-LET radiation for a range of parameters including tumourigenicity, saturation density, doubling time, ability to reconstruct foci on confluent monolayers of untransformed C3H10T½ cells and chromosome complement. These properties were examined for differences between high- and low-LET radiation transformation, between tumourigenic and non-tumourigenic cells derived from foci and between the cell lines developed from the foci and cells isolated from tumours induced by the foci in C3H mice. Although the C3H10T½ cell transformation assay is widely used for the assessment of transformation frequencies a comprehensive examination of the transformed foci has not been done for radiation transformed cells and only in a few studies of chemically transformed cells. Transformation frequencies of low doses of low-LET radiation and the influence of varying dose-rate was also assessed.

Chapter two

Materials and methods

The C3H10T½ cell line (parental untransformed cells, focus and tumour cells) was used for all experiments in this thesis. The parent C3H10T½ cell line was kindly provided by Dr. Lui Hieber, Institur für Strahlenbiologie, Ludwig Maximilians Universitat München, Germany. All C3H10T½ cell lines derived from foci were developed in this laboratory and all tumour cell lines at St. Andrew's University. The protocols described below apply to all the C3H10T½ cell lines, unless otherwise specified. The reagents and equipment used for the following protocols are described in more detail in the appendix.

C3H10T½ cell culture medium

Cell culture medium is prepared by adding 20ml L-glutamine, 110ml of heat inactivated foetal calf serum and 0.5ml gentamicin to one litre of BME medium. This forms 10% serum supplemented BME. 5% serum supplemented BME is made as above except only 55ml of heat inactivated foetal calf serum is added. Medium in the following sections refers to complete medium with L-glutamine, gentamicin and 10% heat inactivated foetal calf serum, unless otherwise stated.

Heat inactivation of serum

Foetal calf serum is supplied without heat inactivation. The serum to be heat inactivated is thawed and then heated to $55^{0}C \pm 1^{0}C$ and held at that temperature for thirty minutes, inverting the bottles of serum at regular intervals. A sample temperature control is always used, consisting of a similar bottle to that containing the serum with a thermometer measuring the temperature of the liquid in the centre of the bottle.

Serum testing

Serum quality can vary greatly between batches and this can adversely affect the growth of the C3H10T½ cells. It is important to assess the effect of a new serum on cell growth and, if possible, to test a variety of sera and select one batch of serum for each series of experiments. All samples of sera to be tested are heat-inactivated as described previously and growth medium prepared for each serum batch. A plating

efficiency test and growth curve is set up for each serum using a single source of C3H10T½ cells. Optimal plating efficiencies between 50% and 60% and a uniform colony size is desirable. The sera with optimal cell plating efficiencies and growth are then tested for spontaneous transformation frequency. Approximately one hundred 175cm^2 culture vessels are set up (seeded with C3H10T½ cells to obtain a viable cell density of approximately 2 cells/cm^2), per serum tested, for the transformation assay. The upper limit of spontaneous transformation frequency per viable cell expected is 3 x10^{-5}. Calculation of transformation frequency is outlined in chapter three.

Preparation of stock cultures

The cell stocks are stored in liquid nitrogen in a cryostat. Protective equipment must be worn when working with liquid nitrogen. The vial of cells is removed from the cryostat and thawed quickly in a water bath at 37^{0}C. The cells are then pipetted into a culture vessel containing warm (approximately 37^{0}C) medium. The culture is incubated at 37^{0}C in an atmosphere of 5% carbon dioxide in air and left for a few hours to allow the cells to attach to the growth surface of the flask. The medium is then replaced with fresh medium to remove the DMSO (dimethyl sulfoxide) added before freezing (see section on freezing C3H10T½ cells). Once the cells are deemed to have recovered from the freezing / thawing procedure (majority of the cells attached to the flask surface and dividing), they are subcultured into 75cm^2 tissue culture flasks.

Preparation and cell counting of stock cell suspensions

Most protocols using the C3H10T½ cells require a single cell suspension of a known cell concentration. A single cell suspension is prepared as follows: the growth medium is removed from the culture and the cells washed with trypsin-EDTA (37^{0}C) to remove any remaining medium. Fresh trypsin-EDTA (37^{0}C) is added to cover the growth area and the culture incubated at 37^{0}C. When the cells have detached from the growth surface (usually within five minutes), the suspension is gently aspirated to separate the cells and mixed with an equal volume of growth medium (the serum neutralises the effect of the trypsin). This single cell suspension is counted by

diluting a volume of cells in isoton (0.5ml cell suspension added to 19.5ml isoton (1:40 dilution)) and counting the cells using the Coulter Counter ZM. Four counts are routinely done (total of 0.5ml counted) and the number of cells per millilitre determined by multiplying the total of the four counts by twenty to take account of the dilution of the cells in isoton.

Freezing C3H10T½ cells

A single cell suspension is prepared and counted. The suspension is centrifuged at 1000 RPM for 5 minutes, and the supernatant discarded. Cells are resuspended in growth medium to give a concentration of at least 8 x 10^5 cells per millilitre. Each cryostat vial is filled with 1ml of cell suspension and 0.1 ml of DMSO (dimethyl sulfoxide). Vials are then placed in the Nicool LM 10 freezing apparatus which was set to decrease temperature linearly to -120°C at a rate of 1°C per minute. Vials are then transferred and stored in the cryostat.

Plating efficiency and survival assay

The survival assay measures the ability of a cell to replicate indefinitely, given the appropriate conditions. A radiation treated cell which can produce a colony in culture of at least fifty cells is deemed to have survived the radiation treatment. The fraction of unirradiated cells which will produce colonies of sufficient size is termed the plating efficiency (see figure 2.1. for sample spreadsheet of plating efficiency calculations). The surviving fraction of cells after treatment with radiation is the fraction of cells which produced colonies divided by the plating efficiency. To set up a survival assay the cells to be irradiated are seeded into 25cm^2 flasks a few days prior to irradiation. The cells are X-irradiated and soon after irradiation cells were harvested, a single cell suspension prepared and the cell concentration determined. Cells are seeded into the culture vessels containing warm (approximately 37^{0}C) pregassed medium at numbers estimated to give one viable cell per square centimetre. Cultures are incubated at 37^{0}C in an atmosphere of 5% carbon dioxide in air for ten to fourteen days. Colonies are stained with methylene blue for fifteen minutes. Flasks are washed

three times with tap water followed by a final rinse in distilled water. The number of cell colonies (with at least fifty cells per colony) are counted and the plating efficiency and surviving fraction determined.

PLATING EFFICIENCY

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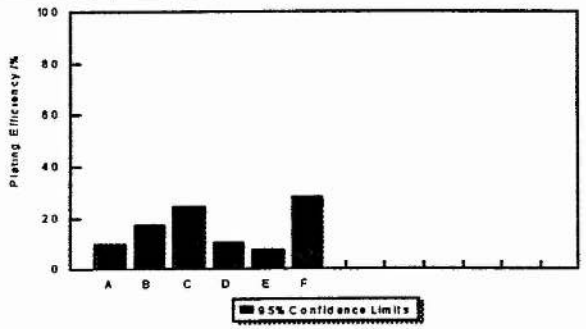
29-Mar-96

Experiment: Sample spreadsheet



Label	Experimental Conditions	Flask Area /cm2	Number of Cells Seeded per Flask	Viable Cell Density /cm-2	Numbers of Colonies Scored per Flask (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	Number of Colonies Mean	Std. Dev.	No. of Flasks	Plating Efficiency /% (± Standard Error)
A		80	499	0.59 ± 0.01	46	47	49	45	50						47.4	2.074	5	9.50 ± 0.19
B		80	506	0.98 ± 0.06	84	66	82	92	66						78	11.58	5	15.42 ± 1.02
C		75	353	1.05 ± 0.05	72	74	93	77	88	68					78.67	9.75	6	22.29 ± 1.13
D		75	356	0.43 ± 0.02	25	35	35	25	25	38	38	38	29	36	32.4	5.739	10	9.10 ± 0.51
E		75	352	0.32 ± 0.02	22	26	22	23	20	25	32	19			23.63	4.104	8	6.71 ± 0.41
F		175	507	0.75 ± 0.02	131	127	126	136	121	150					131.8	10.23	6	26.00 ± 0.82
																	0	
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Figure 2.1. Sample spreadsheet showing number of colonies of cells produced by different cell lines (A to F) on culture dishes of different surface areas and the plating efficiencies calculated.

Growth curves

Growth curves of C3H10T½ cells are used to determine the lag phase, doubling time and saturation density of the cells. The appropriate culture of cells is trypsinised, a single cell suspension prepared and the cell concentration counted. These cells are seeded into 35mm x 10mm petri dishes at approximately six thousand cells per dish. Twenty dishes are seeded per growth curve and two replicate dishes counted daily. Cultures are incubated at 37°C in an atmosphere of 5% carbon dioxide in air. Results are plotted as cell number per dish versus time and the aforementioned parameters calculated. Data obtained and a sample growth curve are presented in section 5.4. All cell lines (focus, tumour, untransformed) were assayed in batches with each batch of cell lines thawed and subcultured together so that all cell lines received similar subculture treatment before the growth curves were set-up.

Radiation Treatment

Most of the irradiations were made using a 420 kVp X-ray machine. This is a standard constant potential X-ray unit and is usually operated at a voltage of 250kV and a current of 15 mA. Irradiations are done using filtered beams (filter containing 1.2mm of aluminium and 0.3mm of copper) and the flask of cells are placed in a perspex phantom, designed to hold a 25 cm^2 culture flask. The dose-rate is altered by reducing the beam current and the distance between the sample and source.

The alpha-particle source used for some irradiations is located at the Medical Research Council Radiobiology Unit, Didcot, England. Details of the source have been published (Roberts and Goodhead 1987, Goodhead *et al.* 1991). The incident alpha-particle energy is 3.26 MeV ± 0.22 MeV and the incident Linear Energy Transfer (LET) in water is 121 keV/μm. The alpha - particles traverse a path of 65mm of helium at atmospheric pressure, a containment - chamber window of 0.35mg cm^{-2} Hostaphan (polyethylene terephthalate: Hoechst) and 3mm of air before entering the base (0.35mg cm^{-2} Hostaphan) of the culture dish on which the cell monolayer is growing. Up to ten dishes are mounted in a horizontal wheel, which is rotated continuously at 3 rev min^{-1} until the required dose has been delivered (Roberts and Goodhead 1987).

Transformation assay

The assay measures the ability of a cell to become transformed *in vitro*, under the appropriate growth conditions. Untransformed C3H10T½ cells grow in culture to form a confluent monolayer on the growth area of a culture vessel. Once confluent, the cells become contact-inhibited and no longer divide. This monolayer is maintained by weekly medium changes. Transformed cells are not contact-inhibited and continue to grow and the subsequent clone of cells is known as a focus. The number of foci per cell seeded is used to determine the transformation frequency.

The C3H10T½ cells to be irradiated are usually subcultured, a few days prior to irradiation, into 25cm^2 culture flasks for X-ray treatment. After irradiation the cells are harvested, a single cell suspension prepared for each dose or control flask, and the cell concentration determined. Cells are seeded into culture flasks containing prewarmed and pregassed medium at numbers aimed to provide two viable cells per square centimetre. Parallel flasks to determine survival are also set up. All cultures are placed at 37^{0}C in an atmosphere of 5% carbon dioxide in air. Cells are stained after ten to fourteen days, depending on growth, and plating efficiency and surviving fractions determined. The viable cell density in the transformation flasks is then calculated.

In the transformation assay medium is changed first at two weeks and then at weekly intervals. The first medium change is with medium supplemented with 10% foetal calf serum, all subsequent changes are with medium supplemented with 5% foetal calf serum. The standard transformation assay continues for six weeks after seeding cells. Flasks are then stained for examination using the following protocol. Growth medium is removed and flasks washed with 1X PBS buffer which removes any remaining serum. Cultures are then fixed in methanol for twenty minutes. The methanol is poured off and the cultures stained with 10% Giemsa (filtered and freshly prepared solution) for twenty minutes. Cultures are washed three times in tap water followed by a final rinse in distilled water. Transformation data are presented in chapters three and four.

Chromosome preparations

Cultures used for chromosome studies are prepared when the cells are in the log phase of growth. Colcemid is supplied in powder form to which 1X PBS is added to form a stock solution of 100μg/ml. This stock (100μg/ml) is diluted one-in-ten to make a working solution of 10μg/ml. 0.2ml of the working stock is added to 20ml of growth medium in $75cm^2$ culture flasks (colcemid concentration of 0.1μg/ml of medium) for approximately four hours. The cells are then harvested using trypsin-EDTA and a single cell suspension prepared. The suspensions are centrifuged at 2000 r.p.m. for five minutes and the supernatant removed and discarded. Cells are resuspended in 5ml of 1% trisodium citrate and incubated at 37^0C for ten minutes, after which the cells are centrifuged as before and the supernatant discarded. Cells are quickly resuspended in freshly prepared fixative (3:1 ethylalcohol : glacial acetic acid). The fixation step takes place at room temperature for ten to fifteen minutes. Cells are centrifuged again as before and the fixation step and centrifugation repeated twice. After the final centrifugation the supernatant is removed and the cells resuspended in the remaining liquid. The cell suspension is dropped onto a slide and allowed to spread. As the preparation dries, the nuclear membrane bursts and the chromosomes spread. Once the slides are dry, they are stained with 10% Giemsa (filtered, freshly prepared solution) for ten to fifteen minutes, washed with tap water, and finally rinsed in distilled water and allowed to dry before examination. Cytogenetics data are presented in chapter 5.5. All cell lines (focus, tumour, untransformed) were assayed in batches with each batch of cell lines thawed and subcultured together so that all cell lines received similar subculture treatment before the cytogenetics studies were carried out.

Tumourigenicity Testing

All foci isolated from the C3H10T½ assay are tested for their ability to produce tumours in C3H mice. The tumourigenicity testing is carried out at St. Andrew's University, Scotland. C3H female mice (four to five weeks old) receive a whole body radiation dose of 5 Gy gamma rays the day before injection of the focus or control C3H10T½ cells to be tested. Four million cells are injected subcutaneously

per mouse and each cell line is tested in four to six mice. The site of injection is examined weekly for tumour formation. Tumour size is graded on a scale of one to six, corresponding to a tumour diameter of 2mm to 12mm. Tumours selected for development of a cell line, are excised and dissected lengthways and a sample removed for culture, the remainder of the tumour is fixed in 10% formal saline for histological examination. Tumourigenicity data are presented in chapter 5.2. All cell lines (focus, untransformed) were assayed in batches with each batch of cell lines thawed and subcultured together so that all cell lines received similar subculture treatment before testing for tumourigenicity.

Focus Reconstruction Studies

The aim of these studies is to test the ability of the focus and tumour cells to form foci when seeded with untransformed C3H10T½ cells. Untransformed cells are seeded in 175 cm^2 culture flasks at 10^4 cells per flask and the cultures allowed to grow to confluence (final cell numbers of approximately five million untransformed cells per flask). When the cultures are confluent, half of the cultures are trypsinised, but the cells are not removed from the flasks. Instead the flasks are seeded with focus or tumour cells or untransformed control cells at a density of approximately one viable cell per centimetre and fresh medium added. At the same time the remaining flasks of confluent cultures (not trypsinised) are also seeded with the focus or tumour cells. Equal numbers of flasks of cells were set-up for the two cell seeding protocols per cell line (thirty to forty flasks per cell line). Cultures are medium changed weekly with 5% serum supplemented medium for four weeks (after addition of focus / tumour cells). Four weeks in culture after confluence is deemed to be sufficient time for development of foci on the monolayers from results of the assay standardisation experiments presented in chapter three. Cultures are then stained as described for the transformation assay and examined. Parallel flasks are seeded to determine the viability of the cells. Focus reconstruction data are presented in chapter 5.3. All cell lines (focus, tumour, untransformed) were assayed in batches with each batch of cell lines thawed and subcultured together so that all cell lines received similar subculture treatment before assessment of the ability to reconstruct foci.

Appendix

Materials for routine culture

Eagle's Basal Medium (BME) with Earle's salts, without L-glutamine (Gibco Life Technologies)

200mM L-glutamine (Gibco Life Technologies)

Foetal Calf Serum (FCS) (Gibco Life Technologies)

50 mg/ml Gentamicin stock (Gibco Life Technologies)

Trypsin-EDTA: 0.25% trypsin with 0.02% EDTA (ethylenediaminetetracetic acid) (Imperial Laboratories)

Dimethyl sulfoxide (DMSO) (Sigma Chemical Company)

Dulbecco's Phosphate Buffered Saline (PBS) without calcium or magnesium (Gibco Life Technologies)

Isoton (Coulter Euro Diagnostics Isoton II)

Giemsa stain (BDH)

Methylene Blue stain (BDH)

25cm^2, 80cm^2, 175cm^2 Tissue culture flasks (Nunclon)

35mm x 10mm Tissue culture petri dishes (Corning)

Equipment

Nicool LM 10 freezing apparatus (Jencons Scientific Ltd.)

Temperature control equipment (Cryostat) (Jencons Scientific Ltd.)

Philips 420 kVp X-ray machine (Ago Installations Ltd.)

Class II Microbiological Safety Cabinet (Envair Limited) with unidirectional laminar downflow

Coulter Counter (Coulter Scientific Instruments), ZM model

Wifug laboratory centrifuge, model 500E

Perspex Gas Boxes supplied with 5% carbon dioxide in air, maintained in a room which is temperature controlled at 37^{0}C.

Additional materials for chromosome studies

Colcemid / demecolcine / N-Deacetyl-N-methylcolchicine (Sigma Chemical Company)

Trisodium citrate (dihydrate) (Sigma Chemical Company)

Ethylalcohol (Hayman Limited), Glacial acetic acid (Sigma Chemical Company)

Chapter three

Low-LET radiation transformation

List of tables and figures

The C3H10T½ transformation assay has been extensively used to determine transformation properties of a variety of agents by several laboratories. The transformation assay relies on the ability of transformed cells to overcome routine growth regulatory controls and continue growing to form multilayers of cells which constitute foci appearing on a continuous monolayer of untransformed C3H10T½ cells (Reznikoff *et al.* 1973). These foci present with a variety of morphological characteristics which are discussed in greater detail in chapter five. The number of foci observed in the C3H10T½ transformation assay is used to calculate transformation frequencies which are then used for comparison of different carcinogens or assessment of the dose-response relationship of a particular carcinogen.

The area of most interest in radiation transformation studies is the low dose region. Table 3.1 presents examples of the range of transformation frequencies published for a dose of 2 to 3 Gy X-rays. The range of radiation qualities and irradiation conditions used makes comparison of data between laboratories difficult. However differences within a particular laboratory can be observed over a number of years. For example, compare the data published by Miller *et al.* in 1989 with data published in 1995 using the same radiation source. In order to obtain reliable data at low doses a large number of transformants must be examined to reduce statistical variation. For this reason a collaboration of six European laboratories was organised in 1990 to standardise the C3H10T½ assay and carry out collaborative experiments with the specific aim of examining the dose-response relationship for transformation by low doses of radiation.

Table 3.1. Examples of transformation frequencies published for 2 to 3 Gy X-rays

Authors	X-ray details	Dose (Gy)	Transformation frequency per viable cell ($x10^{-3}$)	Transformation frequency per viable cell ($x10^{-3}$) per Gy
Miller and Hall 1978, 1979	300 kVp, 12mA, 0.2mm Cu filter	2	~ 0.3 ± 0.07	~0.15
Han and Elkind 1979	50kVp, 20mA, 0.18mm Al filter	2	0.6	0.3
Clark *et al.* 1981	100kVp, 10mA, 0.8mm Al filter	2	0.1 ± 0.017	0.05
Borsa *et al.* 1984	250kVp, 1mm Al filter	2.55	1.65 ± 0.48	0.65
Balcer-Kubiczek *et al.* 1988	240kVp, 15mA, 4mm Cu filter	2	0.454 ± 0.089	0.23
Miller *et al.* 1989	250kVp, 15mA, 0.2mm Cu,1 mm Al filter	2	0.297	0.15
Miller *et al.* 1991	250kVp, 15mA, 0.2mm Cu,1 mm Al filter	3	0.624 ± 0.067	0.21
Saran *et al.* 1991	250kVp, 1.5mm Cu filter	2	0.0706 ± 0.0234	0.035
Cao *et al.* 1993	50kVp, 20mA, 0.18 Al filter	2.5	0.93 ± 0.13	0.37
Miller *et al.* 1995	250kVp, 15mA, 0.2mm Cu,1 mm Al filter	2	0.605	0.3

Collaboration partners

Bettega, D., Calzolari,P. Dipartimento di Fisica, Universita degli Studi di Milano, Via Celoria, 16, I- 20133, Milan, Italy.

Frankenberg, D., Frankenberg-Schwager,M., Bär, K., Pralle, E. Klinische Strahenbiologie und Klinische Strahlenphysik, Georg-August-Universität Göttingen, von Siebold-Straβe 3, 3400, Göttingen, Germany.

Mill, A.J., Allen, L.A., Butler, A., Hall, S.C., Lehane, M. Radiobiology Laboratory, Berkeley Centre, Nuclear Electric Ltd., Berkeley, Gloucestershire, GL13 9PB, United Kingdom.

Hieber, L., Institut für Strahlenbiologie, GSF-Forschungzentrum, Ingolstadter, Landstraβe 1, 85764 Oberschleissheim, München, Germany.

Roberts, C.J., Futter, S., Morgan, G.R. Biotechnology Services, AEA Technology, 353 Harwell Laboratory, Didcot, Oxfordshire, OX11 0RA, United Kingdom.

Saran, A., Pariset, L., Pazzaglia, S. Ente per le Nuove Tecnologie, l'Energia e l'Ambiente, CRE-Casaccia, Settore AMB-BIO, C.P. 2400-00100, Rome, Italy.

Standardisation of the transformation assay

The standard protocol for the C3H10T½ transformation assay is outlined in figure 3.1. As with most biological systems the assay is affected by a variety of influences such as cell density and serum quality. The first series of experiments carried out by the collaboration involved the assessment of the variables in order to minimise their influence on the transformation frequency. Cells were irradiated with 0.25 to 5 Gy X-rays at the Radiobiology laboratories in Berkeley at a dose-rate of 2 Gy per minute (see chapter two for irradiation details) and then transported to the other laboratories where transformation experiments were set up simultaneously in all laboratories forty-eight hours after irradiation. Forty-eight hours was sufficient time to ensure all laboratories had received the cells. During transportation the cells were stored in complete growth medium on melting ice. The optimal cell number for transportation was found to be greater than 10^6 cells /cm^3 since lower cell numbers resulted in reduced plating efficiency after forty-eight hours (Bettega *et al.* 1996). Further experiments revealed that seeding the cells at approximately 2 cells /cm^2 for

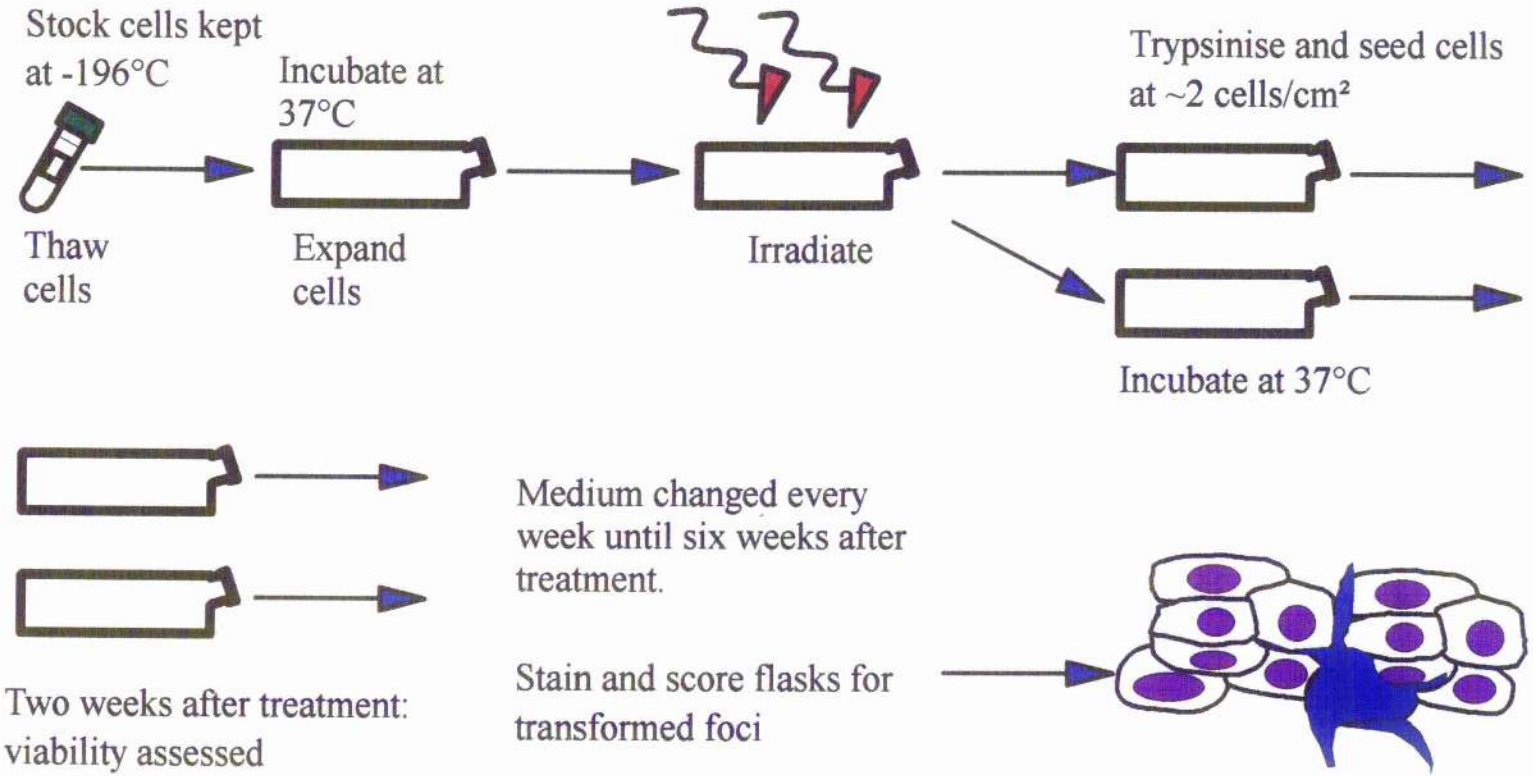


Figure 3.1. Outline of the protocol followed in the setting up and maintaining of C3H10T½ cultures in transformation experiments.

the transformation assay avoided dependence of transformation frequency on seeding density and that the serum used in the growth medium was not a critical factor as long as the serum had been screened as suitable for transformation.

Revision of transformation protocol

The experiments carried out to minimise the effects of variables involved in a collaborative project and the first series of transformation experiments using the standard C3H10T½ transformation protocol outlined in figure 3.1 were completed for the most part by the time the author of this thesis joined the collaboration group. At this stage the need to alter the incubation time in some cases for the transformation assay was recognised and a series of experiments carried out to investigate the influence of time at confluence of the cells on the transformation frequency. The standard assay for transformation of C3H10T½ cells involves a total culture time of six weeks, involving weekly medium changes after the first two weeks (Reznikoff *et al.* 1973). The cells are grown in medium supplemented with ten percent serum for the first three weeks and subsequently the serum content is reduced to five percent for the remaining three weeks.

Figure 3.2 (a) presents the variation of transformation frequency per surviving cell with prolonged incubation of the C3H10T½ cultures in the transformation assay. These preliminary experiments were carried out at the Radiobiology laboratories in Berkeley. Increased transformation frequencies are observed in both the unirradiated control population and the irradiated population with a considerable increase of transformation frequency for the irradiated cells relative to the control population at eight weeks incubation. Based on these results two collaborative experiments were carried out between five laboratories and these results are presented in figure 3.2 (b). It is apparent that the transformation frequency increases for the irradiated population only, although the differences between frequencies at six and eight weeks incubation periods are not significant. These data must be considered in conjunction with the time confluence was reached for the cell cultures and therefore the time the cultures spent at confluence during the transformation assay. In parallel with the above experiments growth curves were obtained to examine when confluence was reached in each

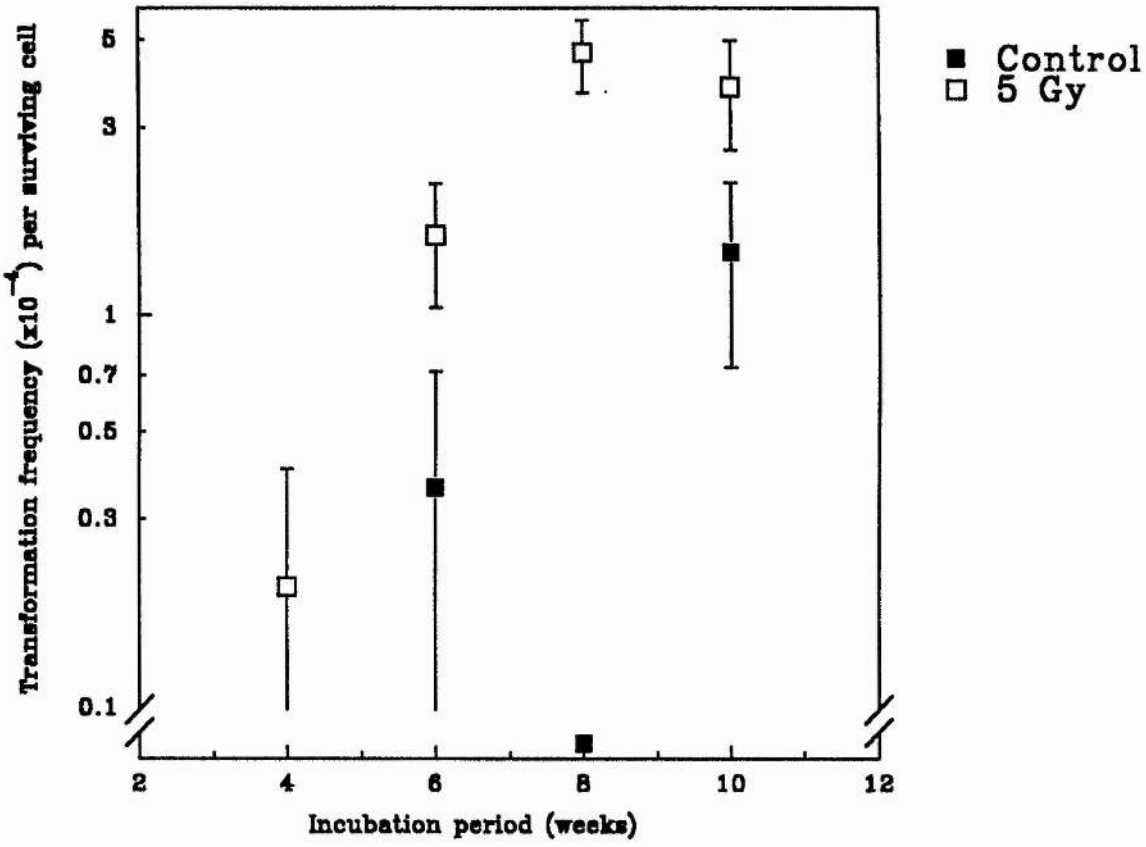


Figure 3.2 (a). Effect of incubation period on transformation frequency (mean ± standard deviation) induced by 5 Gy X-rays. Data are the combined results of preliminary experiments carried out at the Berkeley laboratory only.

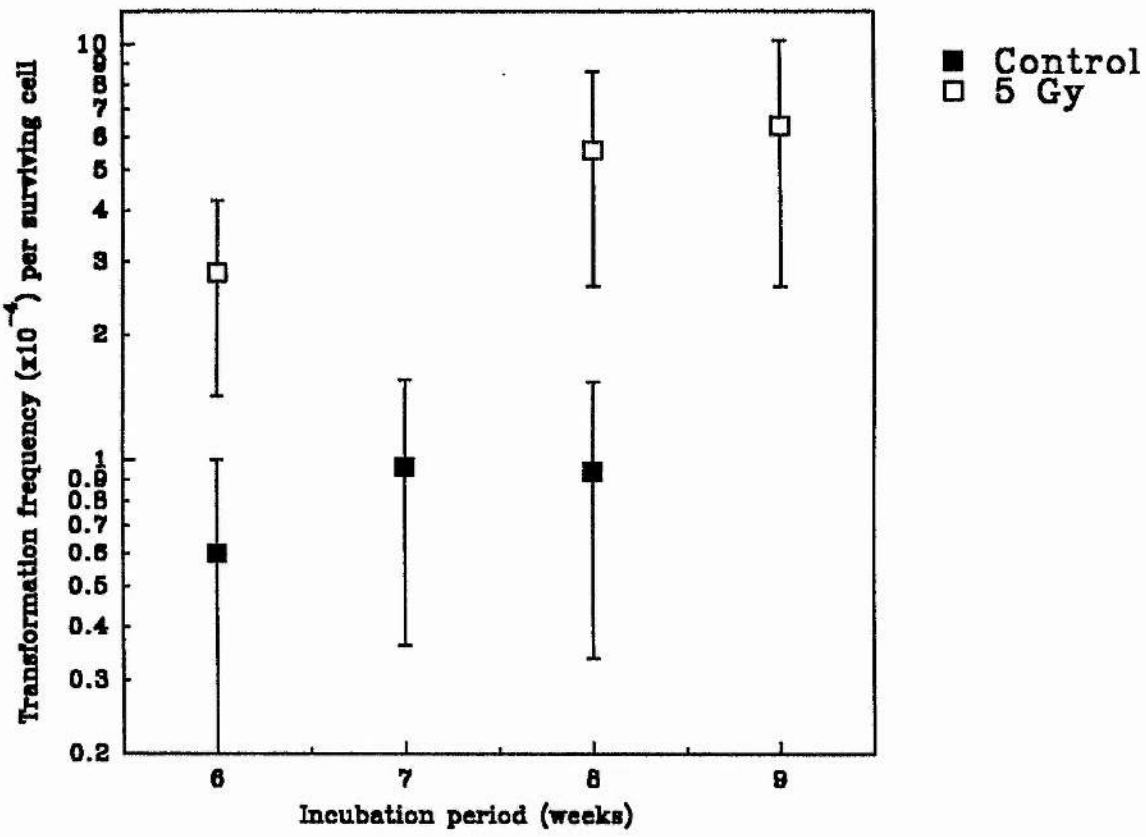


Figure 3.2 (b). Effect of incubation period on transformation frequency (mean ± standard deviation) induced by 5 Gy X-rays. Data are the combined results of individual laboratory data from two collaborative experiments.

laboratory for the untransformed cells and after the cells were irradiated with 5 Gy X-rays (figure 3.3). Figure 3.3 shows that irradiated cultures do take slightly longer to reach confluence at least at high doses. Differences in culture conditions between the laboratories can also result in differences in the time that confluence is reached. Based on these experiments the protocol for the C3H10T½ transformation assay was standardised on the basis of each laboratory ascertaining when confluence is reached in individual experiments for different doses of radiation and incubating the cells for a constant time of four weeks post confluence.

Assessment of transformation frequency

One of the most critical variables in the C3H10T½ transformation assay is the criteria used to categorise the foci as positively or negatively transformed. This topic is dealt with in detail in chapter five (see 5.1). In order to reduce the variation in focus categorisation between laboratories regular meetings were arranged to examine and categorise foci as a collaborative exercise. These meetings were essential to maintain consistency in focus categorisation between the laboratories. For the purposes of the data presented in this chapter only foci scored as positively transformed were included and these appeared in culture as definite type II or III (terms used by Reznikoff *et al.* 1973) with criss-crossing arrays of cells observed on a continuous monolayer of contact-inhibited untransformed C3H10T½ cells. The various categories of foci observed during the collaborative experiments and their comparison with the classes of foci described by Reznikoff *et al.* (1973) are discussed in more detail in chapter five.

Data analysis

The calculation of plating efficiency and surviving fraction are described in chapter two. The transformation frequency is calculated based on the number of positively transformed foci according to the following equation:

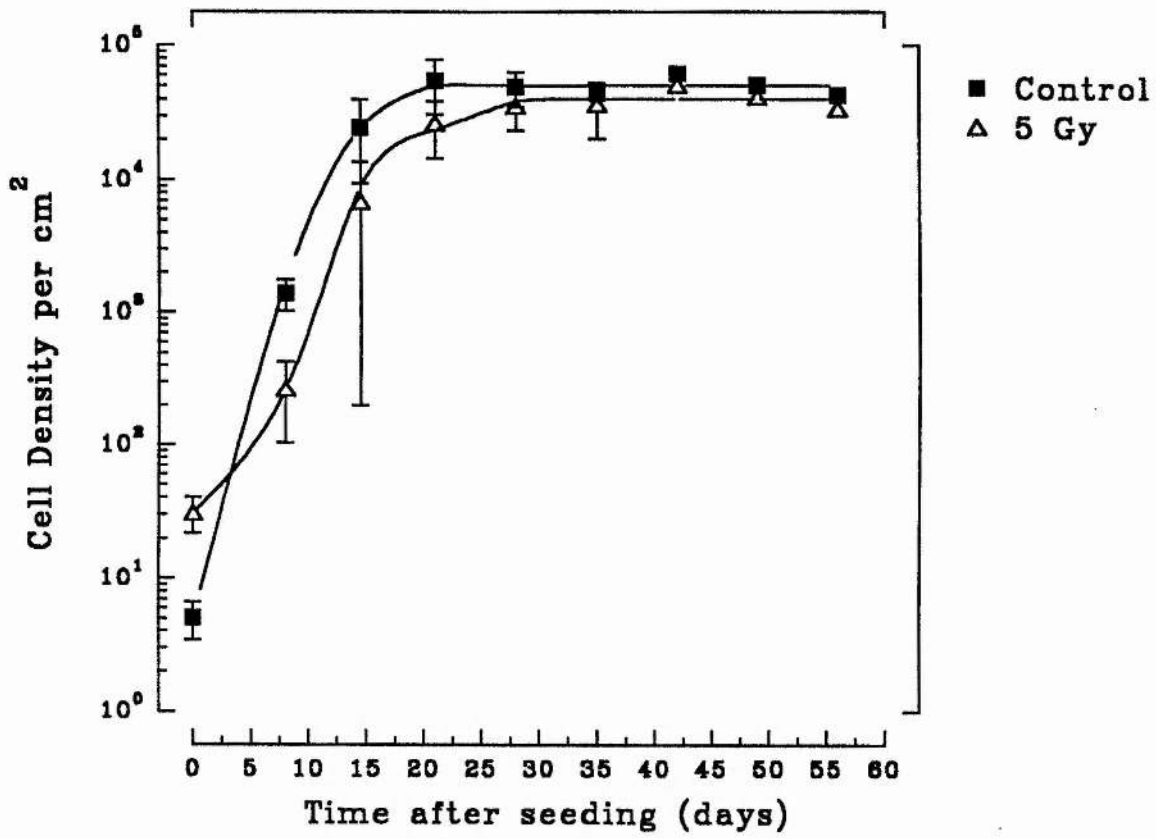


Figure 3.3. Growth curve data. Figure shows the combined growth curve data of five laboratories from two collaborative experiments for the unirradiated control cells and cells irradiated with 5Gy X-rays.

$$TF = \frac{M \times D}{N} \pm \frac{\sqrt{(\frac{M}{D}) \times D}}{N}$$

TF = Transformation frequency

M = Mean number of foci per culture flask

D = Total number of culture flasks

N = Total number of viable cells = Number of cells seeded multiplied by the plating efficiency and the surviving fraction

Calculation of the mean number of foci per culture flask could be done by counting all the foci produced but the potential difficulty with this is that during the experiment transformed cells may break away from the parent focus and produce secondary foci. To avoid double counting of foci Han and Elkind (1979) suggested counting the number of culture flasks with no foci assuming that if the total number of transformed foci is truly Poisson - distributed then the mean number of foci per flask could be estimated from the number of flasks without foci. So the mean number of foci per flask was estimated using the negative log of the fraction of flasks with no foci (Han and Elkind 1979, Brenner and Quan 1990). Thus the revised equation appears as follows:

$$TF = \frac{-\ln(\frac{D_0}{D}) \times D}{N} \pm \frac{(\sqrt{\frac{-\ln(\frac{D_0}{D})}{D}}) \times D}{N}$$

TF = Transformation frequency

D = Total number of culture flasks

D_0 = Number of flasks without foci

N = Total number of viable cells = Number of cells seeded multiplied by the plating efficiency and the surviving fraction

Linear quadratic equations routinely used for survival and transformation analysis were used to calculate lines fitted to the data in the graphs 3.4 to 3.7 and the relationships for the different data, the equations used and the equation parameters calculated are presented in table 3.2. The lines drawn in figure 3.3 for the growth curves were calculated using the equation described in greater detail in chapter 5.4 on the growth parameters of transformed cells.

Table 3.2. Radiation Sensitivity Parameters for Survival and Transformation

Relationship between:	Equation	Parameters		
		α / Gy^{-1}	β / Gy^{-2}	c
Surviving Fraction, *S* and Absorbed Dose, *D*	$S = \exp\{-\alpha.D - \beta.D^2\}$	0.231 ± 0.025	0.0279 ± 0.0066	-
Transformation Frequency, *TF* (6-Week Protocol) and Absorbed Dose,*D*	$TF = c + \alpha.D + \beta.D^2$	(0.626 ± 0.159) x 10^{-4}	(0.036 ± 0.044) x 10^{-4}	(0.596 ± 0.074) x 10^{-4}
Transformation Frequency, *TF* (4-Week Confluence Protocol) and Absorbed Dose, *D*	$TF = c + \alpha.D + \beta.D^2$	(0.919 ± 0.288) x 10^{-4}	NS¶	(0.678 ± 0.118) x 10^{-4}
Transformation Frequency, *TF* (Combined for Both Protocols) and Absorbed Dose, *D*	$TF = c + \alpha.D + \beta.D^2$	(0.838 ± 0.180) x 10^{-4}	NS¶	(0.641 ± 0.079) x 10^{-4}
Transformation Frequency, *TF* (corrected for Spontaneous Frequency) and Absorbed Dose, *D*	$TF = \alpha.D + \beta.D^2$	(0.829 ± 0.084) x 10^{-4}	NS¶	-

¶ not significant (N.S.), values less than 1 x 10^{-10}

The table shows the relationship between the data presented in figures 3.4 to 3.7, equations used to fit the data and the equation parameters calculated from the data.

Transformation frequencies

In total nineteen collaborative experiments were carried out. Data from individual laboratories were combined to produce the survival curve (figure 3.4) and the transformation curves (figures 3.5 to 3.7). Figure 3.5 (a) shows the transformation frequency obtained using both the standard six - week and revised transformation protocols while figure 3.5 (b) presents the combined data of both protocols. Figure 3.6 shows the transformation frequency per viable cell, corrected for the background frequency while figure 3.7 shows the transformation frequency per cell at risk. Combined values were obtained by weighting the individual data of each laboratory by the number of survivors (for transformation) and the number of experiments (for surviving fraction). Individual laboratory data are presented in appendices

3.1 to 3.4.

Figure 3.4 presents the fraction of C3H10T½ cells surviving 0.25 to 5 Gy X-rays. The surviving fraction decreases gradually as the X-ray dose increases with a more rapid decrease in surviving fraction at doses greater than 1 Gy.

Figure 3.5 (a) compares the transformation frequencies obtained using the standard six - week protocol for the transformation assay and the revised protocol for maintaining cultures at confluence for a constant period of four weeks. It is apparent that there is little difference between the transformation frequencies observed using both protocols. The parameters calculated on the line fitted to the data indicates a linear relationship between transformation frequency and dose for both the standard and revised protocols (see table 3.2).

Figure 3.5 (b) presents the transformation frequency per surviving cell when the data presented in figure 3.5 (a) are combined. A linear dose-response relationship is observed and this is more evident when the data are corrected for background frequency and plotted on a linear scale as presented in figure 3.6.

Figure 3.7 presents the transformation frequency per cell at risk. Transformation frequency increases with dose to 1 to 2 Gy where it begins to decrease as transformation saturates and cell killing becomes more dominant.

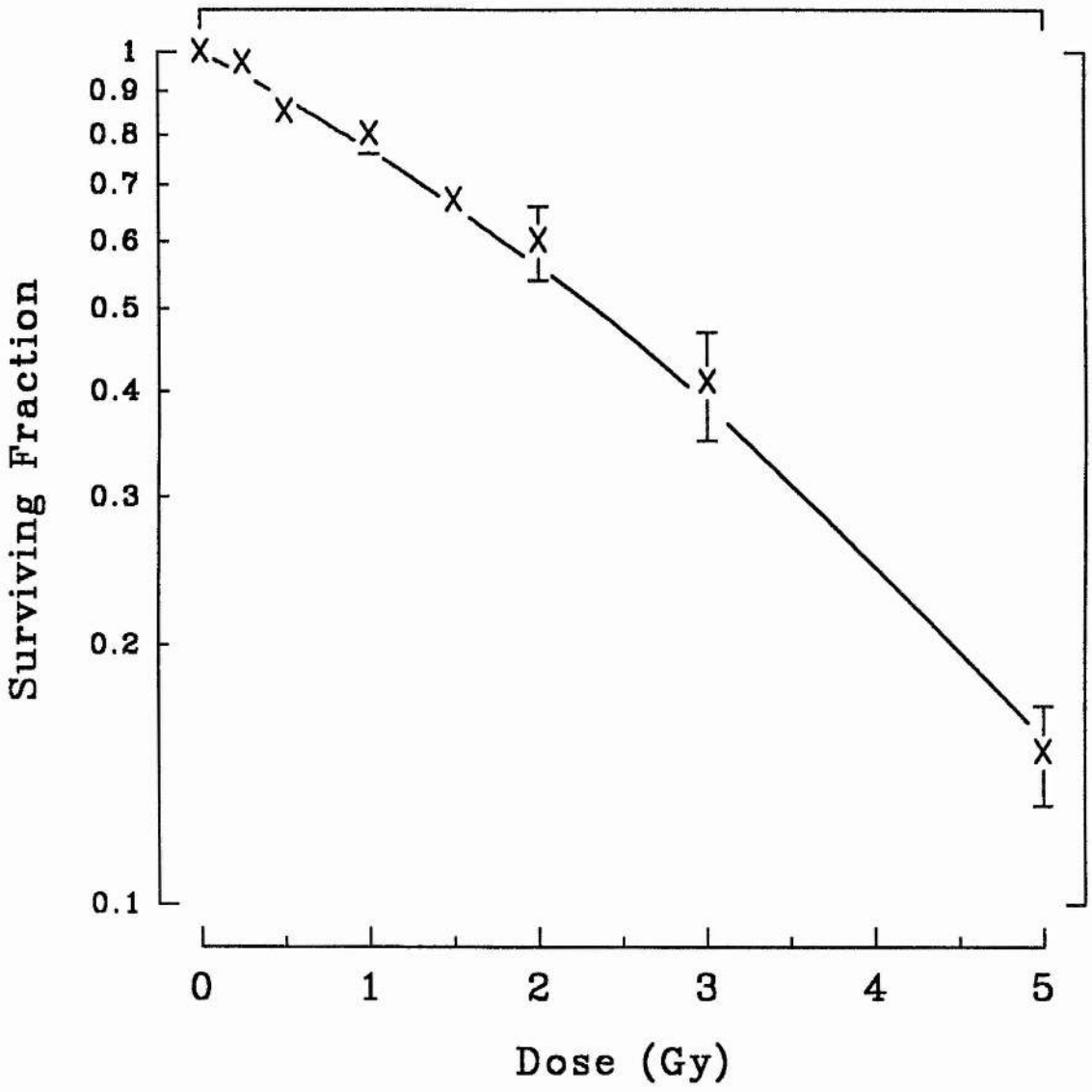


Figure 3.4. Survival data from combined data of all experiments. Individual laboratory data are weighted by the number of experiments. The line was fitted to the data using the equation presented in table 3.2.

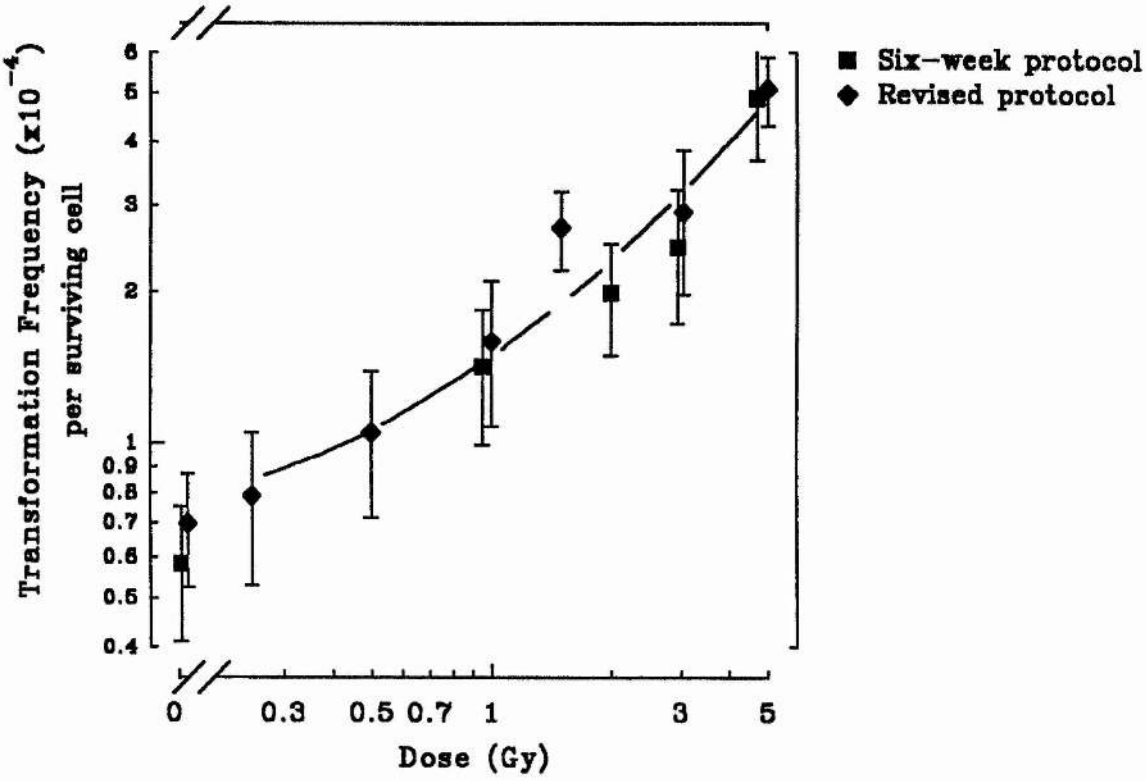


Figure 3.5 (a). Transformation frequencies from combined data for the standard six-week transformation protocol and the revised four-week at confluence protocol. Transformation frequencies from individual laboratories were weighted by the number of survivors in each experiment. The line was fitted to the data using the equation presented in table 3.2.

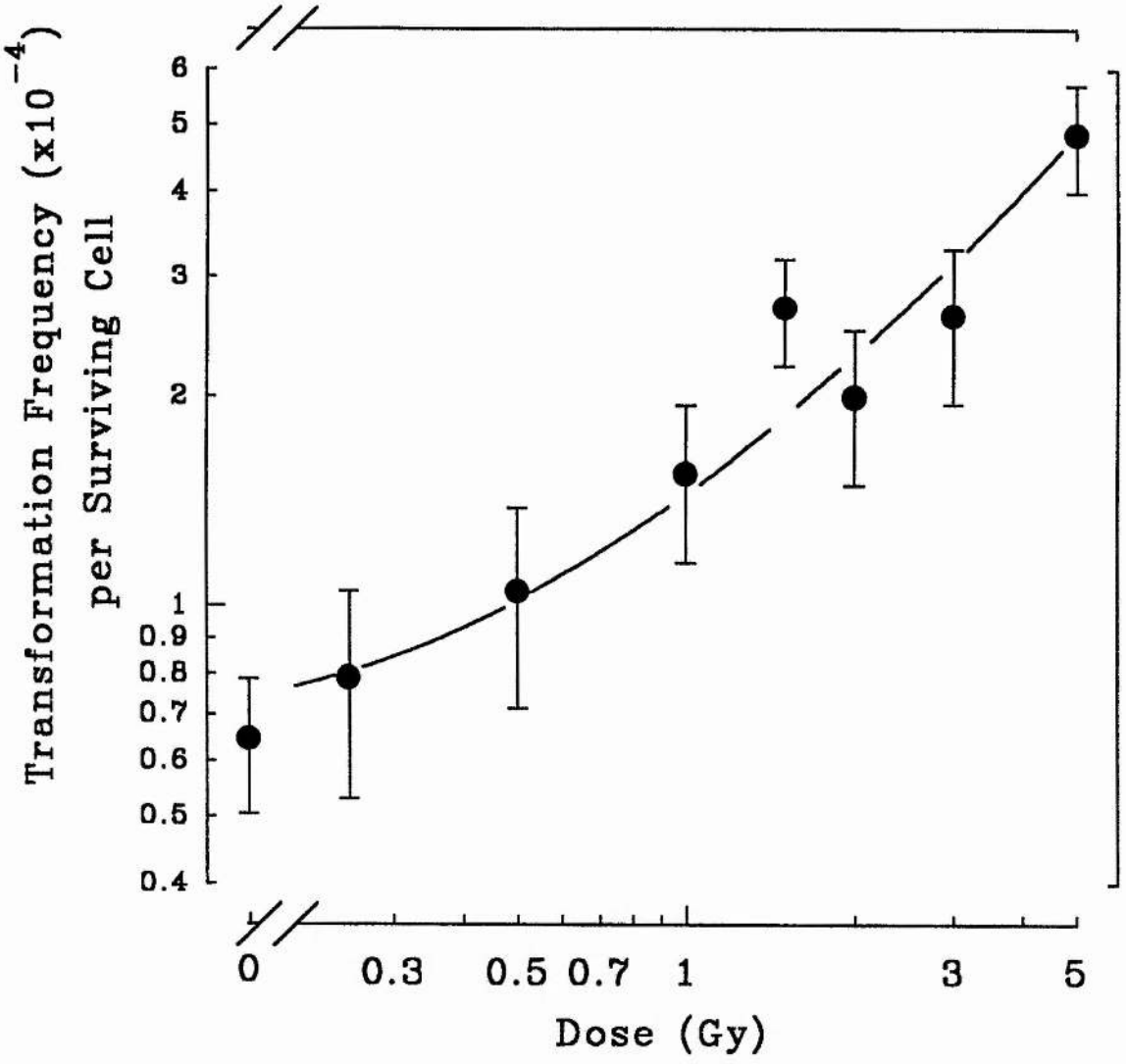


Figure 3.5 (b). Transformation frequencies from combined data for both transformation protocols shown in figure 3.5(a). Transformation frequencies from individual laboratories were weighted by the number of survivors in each experiment. The line was fitted to the data using the equation presented in table 3.2.

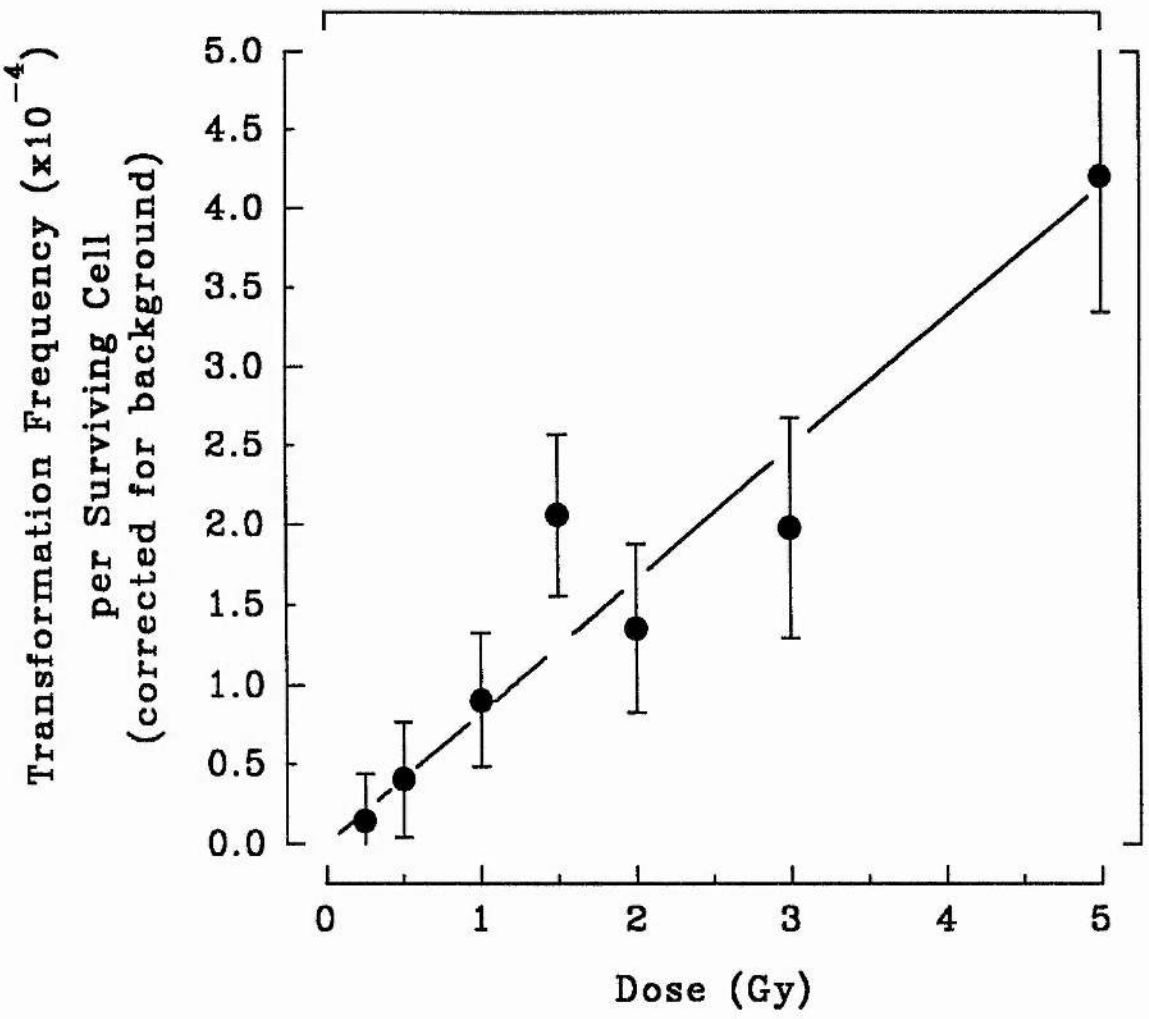


Figure 3.6. Transformation frequencies from combined data for both transformation protocols from all laboratories, corrected for background frequency. Transformation frequencies from individual laboratories were weighted by the number of survivors in each experiment. The line was fitted to the data using the equation presented in table 3.2.

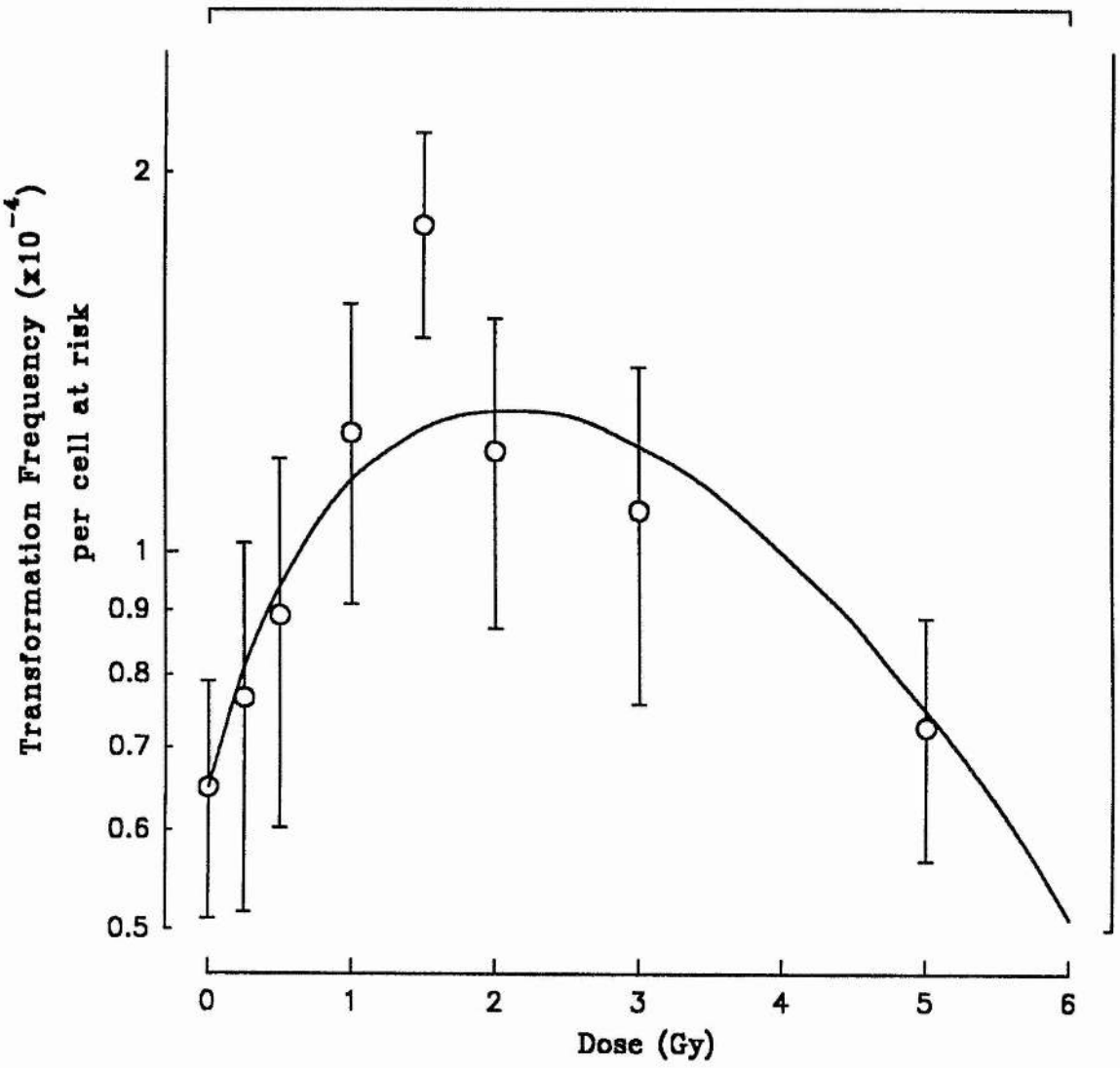


Figure 3.7. Transformation frequencies per cell at risk for the combined data of all experiments using standard and revised transformation protocols. The line was fitted to the data using the equation and values presented in table 3.2.

Discussion

One of the major concerns of exposure to radiation is the risk of cancer induction. Most of the evidence on the carcinogenic effects of radiation has evolved from epidemiology studies of people exposed to higher than average doses of radiation, for example, the atomic bomb survivors. These data relate to high doses and high dose-rates. Since most people will be exposed to low doses and low dose-rates of radiation over a lifetime it is the risk of cancer induction at low doses of radiation that is of prime concern. Calculation of the risk of radiation-induced cancer at low doses involves the assumption of a linear dose-response relationship, that is, that the derived risk is proportional to the dose (UNSCEAR 1993). Cell transformation is the closest *in vitro* assay to carcinogenesis *in vivo* and the transformation data presented in this chapter support the use of linear extrapolation from high to low doses in radiation risk assessment.

Transformation frequency per surviving cell

While a linear dose-response relationship for transformation by high-LET radiation has consistently been established (for example, Hei *et al.* 1988, Hill and Zhu 1991, Miller *et al.* 1989) not all authors agree on a linear relationship between transformation frequency and dose for low-LET radiation. Curvilinear dose-responses have been reported for low-LET radiation by some authors (for example, Hei *et al.* 1988, Hall and Miller 1981, Balcer-Kubiczek and Harrison 1988, Miller *et al.* 1989, Yang *et al.* 1985) while other groups report a linear relationship (for example, Han *et al.* 1984, Hill *et al.* 1987, Borsa *et al.* 1984). Reports on the curvilinear dose-response indicate the response is linear at low doses with a quadratic component at higher doses. The reported dose of X-rays at which the linear and quadratic components meet varies from 0.3 to 1 Gy (Hall and Miller 1981) to 2 to 4 Gy (Balcer-Kubiczek and Harrison 1988). It is important to note that the transformation frequencies presented in this chapter are based on approximately 800 foci from a culture surface area of approximately 300m^2 and thus are supported on a firm statistical basis while data presented in other publications necessarily relies on the efforts and resources of a single laboratory, thus statistical variations may present a problem on smaller data sets. However the important point to note is the general agreement between laboratories of a linear response at low doses of low-LET radiation (less than 1 Gy). The linear response represents cell damage due to single

particle radiation tracks while the quadratic response represents damage due to interaction of lesions from independent tracks (Barendsen 1994).

Transformation frequency per cell irradiated

Transformation frequency is generally presented per cell surviving the radiation exposure although qualitatively a potentially more relevant parameter to radiation risk assessment is the transformation frequency per cell at risk which is presented in figure 3.7. The transformation frequency increases to a maximum at 1 to 2 Gy before transformation saturates and cell killing takes a more dominant role and thus the transformation frequency begins to decrease at higher doses. The dose of 1 to 2 Gy X-rays at which transformation frequency per cell at risk is at a maximum is lower than that reported elsewhere in the literature where it is generally 3 to 4 Gy (Han and Elkind 1979, Little 1977, Hall and Brenner 1992). It is generally agreed that high-LET radiation is a more effective inducer of transformation and the dose of radiation at which maximum transformation frequency per cell at risk is achieved is lower than that for X-rays (for example, Han and Elkind 1979, Hei *et al.* 1988, Hill and Zhu 1991, Hall and Brenner 1992, Miller *et al.* 1989, Yang *et al.* 1985) although again the dose at which maximum transformation frequency per cell at risk for high-LET radiation is achieved varies from 2 Gy (Han and Elkind 1979) to less than 1 Gy (Hall and Brenner 1992). This highlights the variety of data obtained with the same transformation assay utilised in different laboratories under different culture and irradiation conditions.

This chapter concentrates on the quantification of transformation with respect to X-ray exposure. The mechanism(s) by which transformation occurs is dealt with in detail in chapter five where the properties of transformed cells are presented and discussed.

Summary

Standardisation of the C3H10T½ cell transformation assay for use in a collaborative project involved assessment and optimisation of the variables likely to influence the transformation frequency in the different laboratories involved in the project. A single source of radiation was used. Factors examined which may affect the transformation assay included transportation of cells between laboratories, serum quality, cell density both during transportation and at the seeding of the transformation assay, as well as the optimal incubation time of the cultures in the transformation assay once confluence is reached. Maintenance of the cultures at confluence for a constant period of four weeks was adapted to minimise differences in culture conditions between laboratories which may influence the transformation frequency. A linear dose-response relationship down to 0.25 Gy X-rays was observed between transformation frequency and radiation dose using the adapted transformation assay under the culture conditions described in this chapter. Estimation of radiation risk assessments at low doses involves the assumption of a linear dose-response relationship, that is, the derived risk is proportional to the dose and this assumption is supported by the data presented here on radiation-induced transformation *in vitro*.

Appendices

Laboratory	Experiment numbers	Dose /Gy	Average Surviving Fraction	Total Number of Survivors	Total Growth Area /m^2	Total Number of Dishes or Flasks	Total Number of Foci	Trans-formation Frequency per Surviving Cell x 10^4
Berkeley ($162\ cm^2$ Flasks)	4, 5, 8, 9, 10, 11	0	1	120911	7.97	492	3	0.25 ± 0.14
	5, 8, 9	1	0.91	51296	3.99	246	11	2.19 ± 0.65
	5, 8, 9	2	0.71	54497	3.71	229	10	1.88 ± 0.59
	5, 8, 9	3	0.49	36334	3.26	201	8	2.25 ± 0.79
	4, 10	5	0.25	51190	3.82	236	42	9.04 ± 1.33
Göttingen ($75\ cm^2$ Flasks)	4, 5, 7, 8, 10, 11	0	1	63180	5.05	673	2	0.40 ± 0.20
	5, 7	1	0.90	27400	2.14	285	1	0.37 ± 0.37
	7, 8	2	0.37	14200	2.00	266	3	2.12 ± 1.20
	5, 8	3	0.45	15800	1.32	176	2	1.27 ± 0.90
	4, 10, 11	5	0.19	65480	4.32	617	10	1.50 ± 0.47
Harwell ($162\ cm^2$ Flasks)	5, 6, 7	0	1	54674	4.00	247	5	0.92 ± 0.41
	5, 6, 7	1	0.75	46515	3.13	193	5	1.09 ± 0.48
	5, 6, 7	2	0.59	15981	0.83	61	0	<0.63
	5, 6, 7	3	0.36	25839	1.80	111	11	4.48 ± 1.32
Milan ($55/72\ cm^2$ Dishes)	5, 6, 7, 8, 9, 10, 11	0	1	160441	7.73	1301	9	0.56 ± 0.19
	5, 6, 7, 8, 9	1	0.73	64779	6.22	826	11	1.70 ± 0.51
	5, 6, 7, 9	2	0.61	59442	3.58	598	9	1.50 ± 0.50
	5, 6, 7, 9	3	0.41	61116	3.56	594	15	2.50 ± 0.65
	4, 10, 11	5	0.16	52774	2.56	465	22	4.30 ± 0.90
Munich ($55\ cm^2$ Dishes)	4, 5, 6, 7, 8, 10, 11	0	1	151729	9.22	1677	15	0.99 ± 0.26
	5, 6, 7, 8	1	0.85	90955	6.68	1214	22	2.44 ± 0.52
	5, 6, 7, 8	2	0.60	45551	3.52	640	20	4.46 ± 1.00
	5, 6, 7, 8	3	0.38	48372	3.54	644	17	3.56 ± 0.86
	4, 10, 11	5	0.13	77061	4.43	805	43	5.73 ± 0.88
Rome ($55\ cm^2$ Dishes)	4, 6, 7, 8, 9, 10, 11	0	1	95948	6.36	1156	3	0.31 ± 0.18
	6, 7, 8, 9	1	0.84	97826	6.56	1193	3	0.31 ± 0.18
	6, 7, 8, 9	2	0.63	63960	4.51	820	8	1.26 ± 0.44
	6, 7, 8, 9	3	0.41	64880	4.46	811	8	1.24 ± 0.44
	4, 10, 11	5	0.12 ± 0.03	51394	3.95	718	22	4.35 ± 0.92

Appendix 3.1. The table shows the data obtained by individual laboratories for different doses of X-rays using the standard six week protocol for the C3H10T½ transformation assay

Laboratory	Experiment Numbers	Dose /Gy	Average Surviving Fraction	Total Number of Survivors	Total Growth Area /m^2	Total Number of Dishes or Flasks	Total Number of Foci	Trans-formation Frequency per Surviving Cell x 10^4
Berkeley (162/175 cm^2 Flasks)	10, 12, 13, 16, 18, 19	0	1	141877	7.54	436	11	0.79 ± 0.23
	16, 18, 19	0.25	0.96 ± 0.03	258783	12.72	727	23	0.90 ± 0.19
	12, 13, 16, 18	0.5	0.79 ± 0.08	78392	5.27	301	10	1.30 ± 0.41
	12, 13	1	0.80 ± 0.01	52110	3.10	177	12	2.39 ± 0.68
	12, 13	1.5	0.75 ± 0.07	47920	2.70	154	15	3.29 ± 0.83
	16, 19	3	0.59 ± 0.48	61885	2.12	121	12	2.04 ± 0.57
	10	5	0.17	13697	1.02	63	7	5.42 ± 1.99
Göttingen (75 cm^2 Flasks)	10 - 14, 16 - 18	0	1	188506	9.87	1316	20	1.07 ± 0.24
	14, 16 - 18	0.25	0.97	242000	10.83	1444	28	1.16 ± 0.22
	12 - 14, 16 - 18	0.5	0.81	122667	6.50	867	13	1.07 ± 0.30
	12, 13, 17	1	0.67	63357	3.35	446	14	2.25 ± 0.60
	12 - 14	1.5	0.66	61775	3.32	442	21	3.46 ± 0.76
	16, 18	3	0.32	15600	1.00	133	8	5.30 ± 1.90
	10, 11	5	0.17	31365	2.16	288	21	6.95 ± 1.50
Milan (55/72 cm^2 Dishes)	10 - 12, 14 - 18	0	1	191060	7.75	1342	6	0.31 ± 0.13
	14 - 18	0.25	0.90 ± 0.04	266580	11.50	1801	8	0.30 ± 0.11
	12, 14 - 18	0.5	0.88 ± 0.03	115654	5.90	872	4	0.35 ± 0.17
	12, 17	1	0.67 ± 0.12	20979	1.60	293	1	0.48 ± 0.48
	12, 14	1.5	0.77 ± 0.07	28399	1.77	256	7	2.50 ± 0.34
	15, 16, 18	3	0.31 ± 0.02	32352	1.56	283	4	1.25 ± 0.60
	10, 11	5	0.15 ± 0.05	33242	1.41	256	15	4.65 ± 1.20
Munich (55 cm^2 Dishes)	10 - 14, 16 - 18	0	1	195017	8.86	1611	19	0.98 ± 0.23
	14, 16 - 18	0.25	0.92	168600	6.59	1198	27	1.62 ± 0.30
	12, 14, 16 - 18	0.5	0.85	104523	4.90	891	23	2.23 ± 0.47
	12, 17	1	0.87	53524	2.21	401	12	2.28 ± 0.66
	12 - 14	1.5	0.60	57523	3.03	551	18	3.18 ± 0.75
	16, 18	3	0.41	19400	1.20	218	13	6.91 ± 1.92
	10, 11	5	0.095	26058	1.88	341	16	6.29 ± 1.57
Rome (55 cm^2 Dishes)	12 - 14, 16 - 18	0	1	134070	5.70	1036	3	0.22 ± 0.13
	14, 16 - 18	0.25	1.11 ± 0.06	187712	7.66	1392	2	0.11 ± 0.08
	12 - 14, 16 - 18	0.5	0.91 ± 0.09	118957	5.24	952	6	0.51 ± 0.21
	12, 13, 17	1	0.76 ± 0.09	60654	2.73	497	0	<0.17
	12 - 14	1.5	0.62 ± 0.10	54220	2.79	508	5	0.93 ± 0.41
	16, 18	3	0.44 ± 0.06	23490	0.92	167	6	2.60 ± 1.05
	10, 11	5	0.13 ± 0.04	33689	1.79	326	9	2.71 ± 0.90

Appendix 3.2. The table shows the transformation frequencies obtained by individual laboratories for different doses of X-rays using the revised protocol of four weeks in culture post - confluence for the C3H10T½ transformation assay

Laboratory	Experiment Numbers	Dose /Gy	Average Surviving Fraction	Total Number of Survivors	Total Growth Area /m^2	Total Number of Dishes or Flasks	Total Number of Foci	Trans-formation Frequency per Surviving Cell x 10^4
Berkeley (162/175 cm^2 Flasks)	4, 5, 8 - 13, 16, 18, 19	0	1	262788	15.51	928	14	0.54 ± 0.14
	16, 18, 19	0.25	0.96 ± 0.03	258783	12.72	727	23	0.90 ± 0.19
	12, 13, 16, 18	0.5	0.79 ± 0.08	78392	5.27	301	10	1.30 ± 0.41
	5, 8, 9, 12, 13	1	0.87 ± 0.03	103406	7.08	423	23	2.29 ± 0.47
	12, 13	1.5	0.75 ± 0.07	47920	2.70	154	15	3.29 ± 0.83
	5, 8, 9	2	0.71 ± 0.07	54497	3.71	229	10	1.88 ± 0.59
	5, 8, 9, 16, 19	3	0.51 ± 0.05	98170	5.37	322	20	2.10 ± 0.46
	4, 10	5	0.21 ± 0.02	64887	4.84	299	49	8.25 ± 1.13
Göttingen (75 cm^2 Flasks)	4, 5, 7, 8, 10 - 14, 16 - 18	0	1	288986	17.10	2275	24	0.84 ± 0.17
	14, 16 - 18	0.25	0.97	242000	10.83	1444	28	1.16 ± 0.22
	12 - 14, 16 - 18	0.5	0.81	122667	6.50	867	13	1.07 ± 0.30
	5, 7, 12, 13, 17	1	0.79	90757	5.48	731	15	1.67 ± 0.43
	12 - 14	1.5	0.66	61775	3.32	442	21	3.46 ± 0.76
	7, 8	2	0.37	14200	2.00	266	3	2.12 ± 1.20
	5, 8, 16, 18	3	0.39	31400	2.32	309	10	3.24 ± 1.00
	4, 10, 11	5	0.17	96845	6.79	905	31	3.26 ± 0.59
Harwell (162 cm^2 Flasks)	5 - 7	0	1	54674	4.00	247	5	0.92 ± 0.41
	5 - 7	1	0.75	46515	3.13	193	5	1.09 ± 0.48
	5 - 7	2	0.59	15981	0.83	61	0	<0.63
	5 - 7	3	0.36	25839	1.80	111	11	4.48 ± 1.32
Milan (55/72 cm^2 Dishes)	5 - 12, 14 - 18	0	1	351501	15.48	2643	15	0.43 ± 0.11
	14 - 18	0.25	0.90 ± 0.04	266580	11.48	1801	8	0.30 ± 0.11
	12, 14 - 18	0.5	0.88 ± 0.03	115654	5.90	972	4	0.35 ± 0.17
	5 - 9, 12, 17	1	0.71 ± 0.07	85658	7.83	1119	12	1.41 ± 0.41
	12, 14	1.5	0.77 ± 0.07	28399	1.77	256	7	2.50 ± 0.94
	5 - 7, 9	2	0.61 ± 0.08	59442	3.58	598	9	1.52 ± 0.51
	5 - 7, 9, 15, 16, 18	3	0.37 ± 0.03	93468	5.12	877	19	2.06 ± 0.47
	4, 10, 11	5	0.16 ± 0.03	86016	3.97	721	37	4.41 ± 0.72
Munich (55 cm^2 Dishes)	4 - 8, 10 - 14, 16 - 18	0	1	346746	18.08	3288	34	0.99 ± 0.17
	14, 16 - 18	0.25	0.92	168600	6.59	1198	27	1.62 ± 0.30
	12, 14, 16 - 18	0.5	0.85	104523	4.90	891	23	2.23 ± 0.47
	5, 6, 7, 8, 12, 17	1	0.86	144479	8.88	1615	34	2.38 ± 0.48
	12 - 14	1.5	0.60	57523	3.03	551	18	3.18 ± 0.75
	5 - 8	2	0.60	45551	3.52	640	20	4.46 ± 1.00
	5, 6, 7, 8, 16, 18	3	0.40	67772	4.74	862	30	4.51 ± 0.82
	4, 10, 11	5	0.13	77061	4.43	805	43	5.73 ± 0.88

Laboratory	Experiment Numbers	Dose /Gy	Average Surviving Fraction	Total Number of Survivors	Total Growth Area /m^2	Total Number of Dishes or Flasks	Total Number of Foci	Trans-formation Frequency per Surviving Cell x 10^4
Rome (55 cm^2 Dishes)	4, 6 - 14, 16 - 18	0	1	230018	12.28	2192	6	0.26 ± 0.11
	14, 16 - 18	0.25	1.11 ± 0.06	187712	7.66	1392	2	0.11 ± 0.08
	12 - 14, 16 - 18	0.5	0.91 ± 0.09	118957	5.24	952	6	0.51 ± 0.21
	6 - 9, 12, 13, 17	1	0.81 ± 0.05	158480	9.30	1690	3	0.19 ± 0.11
	12 - 14	1.5	0.62 ± 0.10	54220	2.79	508	5	0.93 ± 0.41
	6 - 9	2	0.63 ± 0.05	63960	4.51	820	8	1.26 ± 0.44
	6 - 9, 16, 18	3	0.42 ± 0.03	88370	5.38	978	14	1.60 ± 0.43
	4, 10, 11	5	0.12 ± 0.03	85083	5.85	1044	31	3.70 ± 0.66

Appendix 3.3. The table shows the combined transformation frequencies obtained by individual laboratories for different doses of X-rays using the standard and modified protocols for the C3H10T½ transformation assay. Data are the combined data presented in appendices 3.1 and 3.2.

Dose/Gy	Surviving Fraction	Transformation Frequency per Surviving Cell x10 000				
		Data from 6 Week Protocol Experiments	Data from 4 Week Confluence Protocol Experiments	Data from Both Protocols Combined	Combined Data (corrected for background frequency)	
0	1	0.57 ± 0.13	0.67 ± 0.17	0.66 ± 0.12	0	Unweighted Average
0.25	0.97 ± 0.04	-	0.82 ± 0.28	0.82 ± 0.28	0.15 ± 0.30	
0.5	0.85 ± 0.02	-	1.09 ± 0.33	1.09 ± 0.33	0.43 ± 0.35	
1	0.80 ± 0.03	1.35 ± 0.37	1.60 ± 0.51	1.51 ± 0.33	0.84 ± 0.35	
1.5	0.68 ± 0.03	-	2.67 ± 0.47	2.67 ± 0.47	2.01 ±0.48	
2	0.59 ± 0.05	1.87 ± 0.60	-	1.87 ± 0.60	1.21 ± 0.61	
3	0.41 ± 0.02	2.55 ± 0.52	3.62 ± 1.07	3.00 ± 0.52	2.34 ± 0.54	
5	0.16 ± 0.02	4.98 ± 1.22	5.20 ± 0.74	5.07 ± 0.90	4.41 ± 0.91	
0	1	0.58 ± 0.17	0.70 ± 0.17	0.65 ± 0.14	0	Average Weighted by Number of Experiments (Surviving Fraction) or Total Survivors (Transformation Frequency)
0.25	0.97 ± 0.04	-	0.79 ± 0.26	0.79 ± 0.26	0.14 ± 0.29	
0.5	0.85 ± 0.02	-	1.05 ± 0.34	1.05 ± 0.34	0.41 ± 0.36	
1	0.80 ± 0.04	1.41 ± 0.42	1.59 ± 0.52	1.55 ± 0.40	0.90 ± 0.42	
1.5	0.67 ± 0.03	-	2.71 ± 0.49	2.71 ± 0.49	2.06 ± 0.51	
2	0.60 ± 0.06	2.00 ± 0.51	-	2.00 ± 0.51	1.35 ± 0.53	
3	0.41 ± 0.06	2.47 ± 0.74	2.91 ± 0.94	2.63 ± 0.67	1.98 ± 0.69	
5	0.15 ± 0.02	4.88 ± 1.20	5.09 ± 0.79	4.85 ± 0.85	5.20 ± 0.86	

Appendix 3.4. Table shows the combined data of all laboratories for surviving fractions and transformation frequencies for different doses of X-rays.

Chapter four

Effect of dose-rate on low-LET radiation transformation

List of tables and figures:

Most of the data available on the biological effects of exposure to ionising radiation relate to high doses and high dose-rates. Information about low doses and low dose-rates is mostly extrapolated back from the high dose data. To determine the risk of radiation-induced cancer at low doses over extended periods of time, it is assumed that the dose-response relationship is linear, i.e., the derived risk is proportional to the dose. However at low doses and dose-rates, there is evidence of a reduced effectiveness, especially for low-LET radiations (data summarised in UNSCEAR 1993). Thus a Dose and Dose-Rate Effectiveness Factor (DDREF) is applied to the risk estimates derived from the high doses and dose-rates. DDREF is defined as the factor by which the slope of a pure linear model fitted to the data should be divided to give the low dose slope, that is, the linear term in a linear quadratic dose-response model. Estimates of DDREF vary from 2 (ICRP 1977, 1990) to 2-10 (UNSCEAR, BEIR). Most radiation received in a lifetime is likely to be chronic low dose exposure, for example, environmental, occupational (nuclear industry workers, radiographers), and medical (multiple low doses of medical X-rays). The effect of lowering the dose-rate of irradiation with X-rays on the survival and transformation of C3H10T½ cells is examined in this chapter.

Experiment details and data analysis

The X-ray source details have been outlined in chapter two and this source was also used to obtain the data presented in chapter three. A constant dose of 3 Gy X-rays was used and the dose was delivered to the C3H10T½ cells at two different dose-rates. The acute dose was delivered in a few minutes exposure at a dose-rate of 0.8 Gy per minute (high dose-rate) while the chronic dose was delivered over a five hour exposure period at 0.01 Gy per minute (low dose-rate). The protocol for the transformation assay was the revised protocol described in chapter three. Assessment of transformation of the high dose-rate data was divided into two parts. In the first part the survival and transformation of the high dose-rate treated cells were assessed immediately after irradiation (referred to as 0 h in the figures) in parallel with unirradiated control cells. The second part of the assessment of transformation was carried out five hours later when the low dose-rate treated cells were being assessed,

at which stage another assessment of the cells irradiated at high dose-rate (referred to as 5 h in the figures) was carried out in parallel with unirradiated control cells.

Calculation of plating efficiencies and surviving fractions are described in chapter two while the calculation of transformation frequency is described in chapter three.

Transformation frequencies and surviving fractions

Figure 4.1 presents the transformation frequency per viable cell exposed to 3 Gy X-rays at high (0.8 Gy/min) and low (0.01 Gy/min) dose-rate. A reduction of the transformation frequency with lowering of the dose-rate is apparent. Although there is no significant difference between the transformation frequencies of cells exposed to high dose-rate when plated immediately compared to plating five hours after irradiation the mean transformation frequencies suggest the possibility of an increase in transformation frequency with delayed plating of the irradiated cells. Thus it was decided to examine this possibility by assessing the transformation frequencies of the cells irradiated at high dose-rate at shorter time intervals after irradiation (0.8 and 1.4 hours after irradiation). Delayed plating of the transformation assay presents no significant difference in transformation frequency of cells irradiated at high dose-rate when the transformation is assessed at 0.8 hours and 1.4 hours after irradiation compared to the transformation frequency of the cells assessed immediately after irradiation and five hours after irradiation (data presented in appendix 4.1). The data from the collaborative project described in chapter three are included for reference.

Figure 4.2 presents the surviving fractions of the C3H10T½ cells exposed to 3 Gy X-rays at high and low dose-rates. An increased survival is noted for the low dose-rate data compared to high dose-rate data (0 h) although the difference is not significant. The data from the collaborative project described in chapter three are included for reference.

Figure 4.3 presents the transformation frequency per cell irradiated. A reduction of the transformation frequency with lowering of the dose-rate is apparent compared to the high dose-rate data as observed for the transformation frequency per viable cell.

Transformation frequency and survival data presented in the above figures are

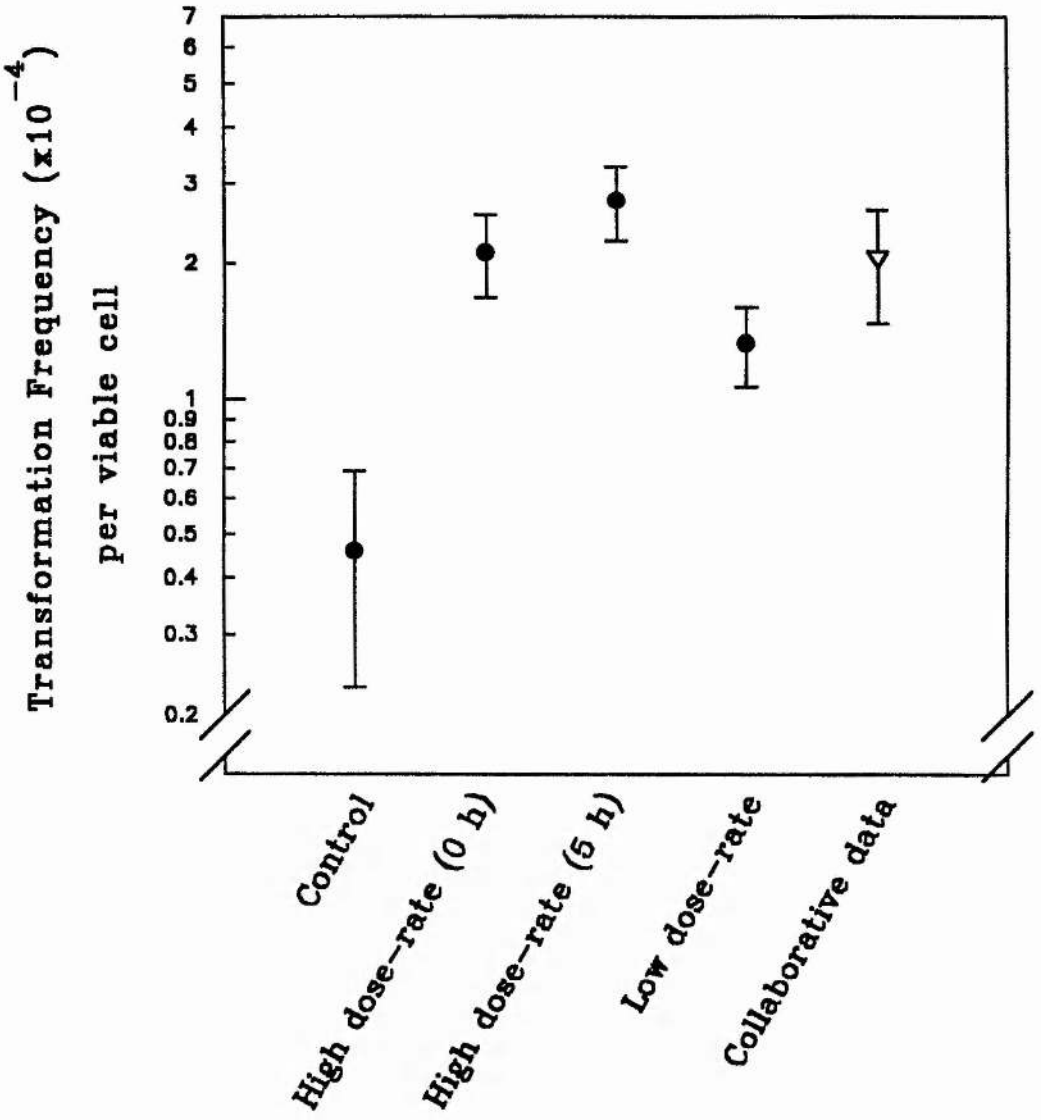


Figure 4.1. Transformation frequency per viable cell exposed to 3 Gy X-rays. Radiation dose was delivered at high dose-rate (0.8 Gy / min) and low dose-rate (0.01 Gy / min). The hours given with the high dose-rate legend indicate the time after irradiation when the transformation assays were set up. Transformation assays were carried out for cultures irradiated with high dose-rate immediately after irradiation (0 h) and in parallel with the cultures irradiated with the low dose-rate (5 h). All high dose-rate irradiations were carried out at about the same time. Collaborative data refer to data obtained by the Berkeley laboratory at high dose-rate presented in chapter three.

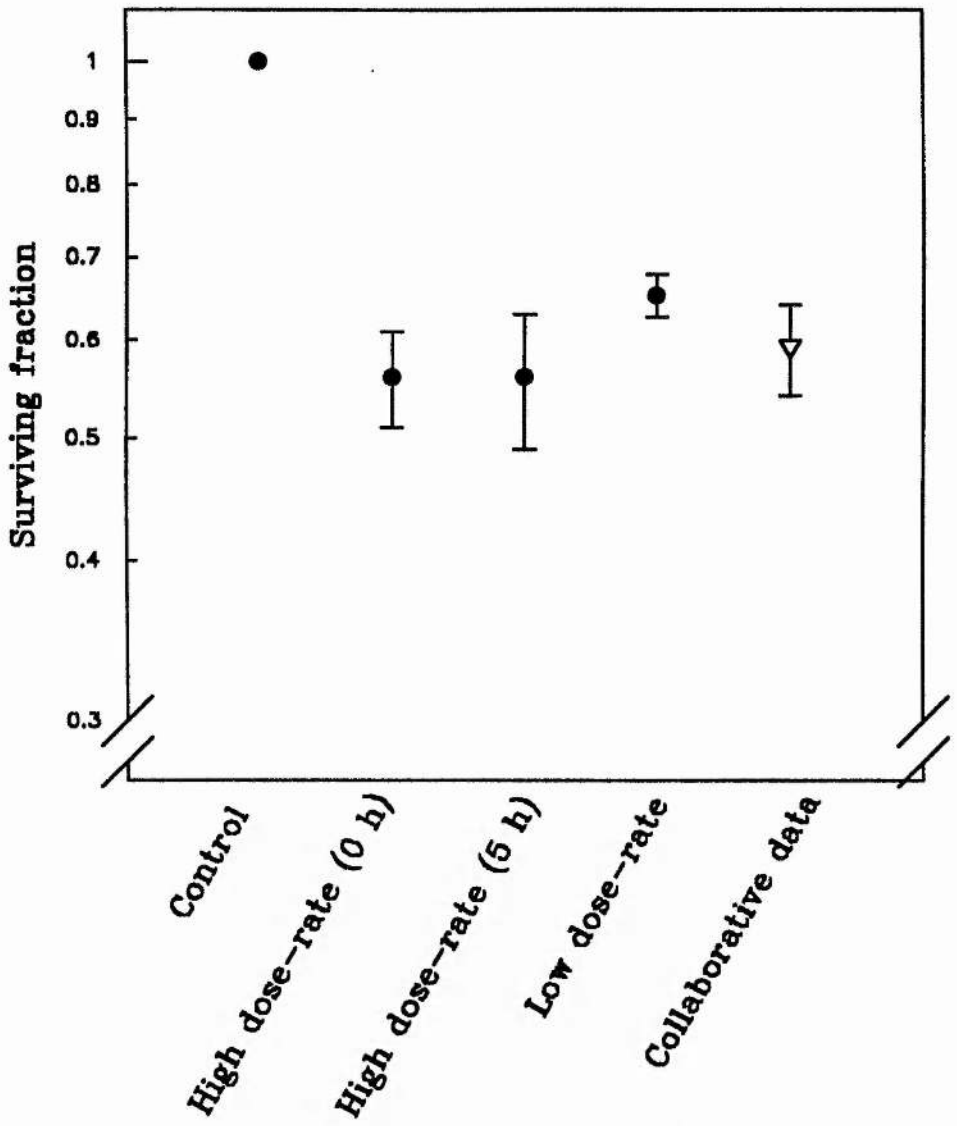


Figure 4.2. Surviving fractions of C3H10T½ cells exposed to 3 Gy X-rays. Radiation dose was delivered at high dose-rate (0.8 Gy / min) and low dose-rate (0.01 Gy / min). The hours given with the high dose-rate legend indicate the time after irradiation when the survival assays were set up. Survival assays were carried out for cultures irradiated with high dose-rate immediately after irradiation (0 h) and in parallel with the cultures irradiated with the low dose-rate (5 h). All high dose-rate irradiations were carried out at about the same time. Collaborative data refer to data obtained by the Berkeley laboratory at high dose-rate presented in chapter three.

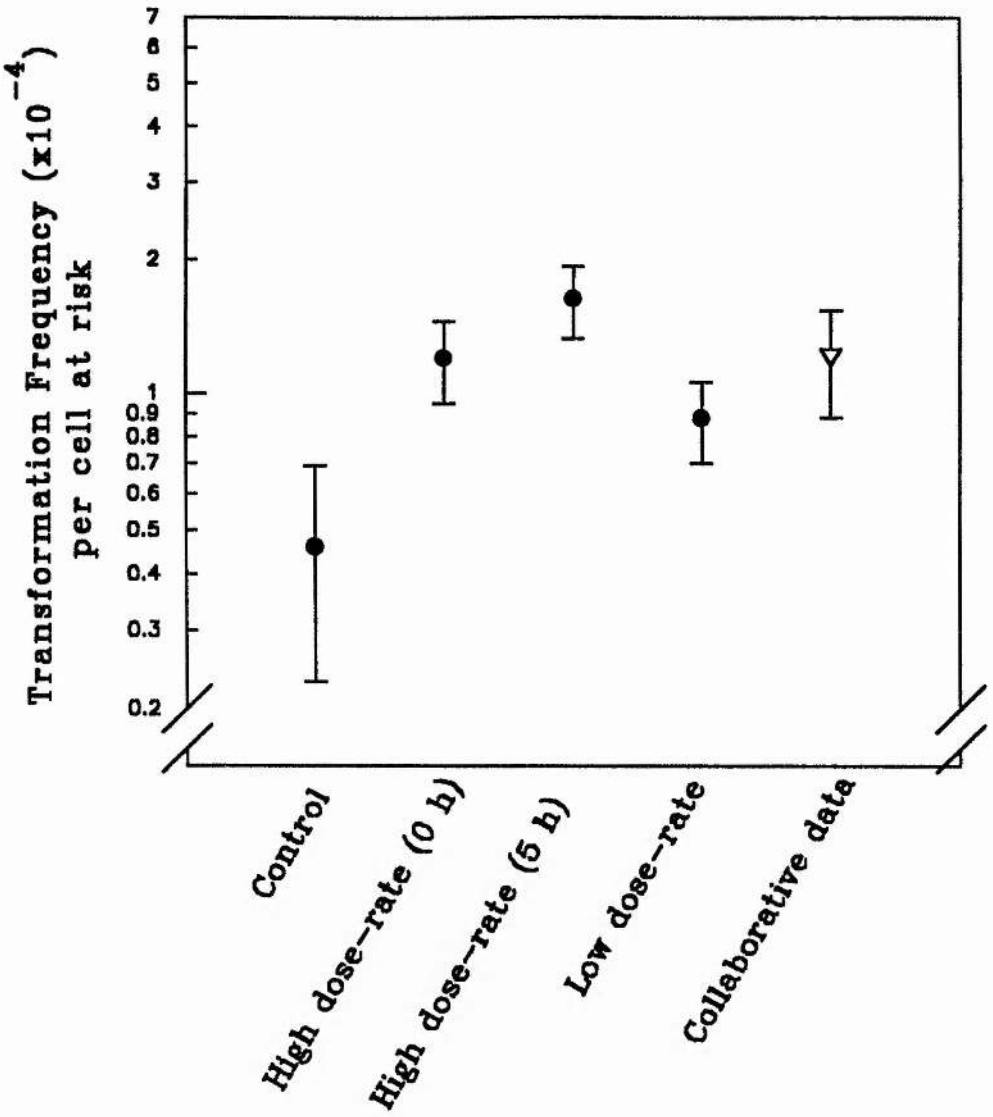


Figure 4.3. Transformation frequency per irradiated cell. Radiation dose was delivered at high dose-rate (0.8 Gy / min) and low dose-rate (0.01 Gy / min). The hours given with the high dose-rate legend indicate the time after irradiation when the transformation assays were set up. Transformation assays were carried out for cultures irradiated with high dose-rate immediately after irradiation (0 h) and in parallel with the cultures irradiated with the low dose-rate (5 h). All high dose-rate irradiations were carried out at about the same time. Collaborative data refer to data obtained by the Berkeley laboratory at high dose-rate presented in chapter three.

also presented in table 4.1. The ratios of the high to low dose-rate data are presented in table 4.2. The ratios were calculated after the transformation frequencies were corrected for background frequency. Individual ratios were calculated for high dose-rate data at the different plating times after irradiation relative to the low dose-rate data and then a ratio of the average transformation frequency at high dose-rate relative to the low dose-rate data calculated. The minimum ratio was calculated for comparison of the transformation frequencies of cells assessed immediately after irradiation while the maximum ratio was calculated when the cells irradiated at high dose-rate were assessed 1.4 hours after irradiation. When the ratios of transformation frequencies of cells irradiated at high dose-rate and not plated immediately after irradiation relative to the data from cells assessed immediately after irradiation were examined the greatest ratios were for cells with delayed plating times of about one hour after radiation treatment.

Table 4.1. Transformation frequencies and survival data

Dose-rate	Number of experiments	Average surviving fraction ± standard error	Total cells seeded	Total surviving cells	Total number of flasks	Total number of flasks with foci	T.F. (x 10^{-4}) per viable cell ± standard error	T.F. (x 10^{-4}) per irradiated cell ± standard error
Control	4	1.0	166746	87209	178	4	0.46 ± 0.23	0.46 ± 0.23
High (0 h)	4	0.56 ± 0.05	656029	111808	378	23	2.12 ± 0.44	1.2 ± 0.25
High (5 h)	4	0.56 ± 0.07	601854	105908	363	28	2.75 ± 0.51	1.63 ± 0.3
Low	4	0.65 ± 0.03	819073	184996	473	24	1.33 ± 0.27	0.88 ± 0.18

Table shows the data presented in figures 4.1 to 4.3. High refers to the high dose-rate of 0.8 Gy per minute and low refers to the low dose-rate of 0.01 Gy per minute. The time given for each high dose-rate is the time after irradiation at which the transformation frequency (T.F.) was assessed.

Table 4.2. Ratio of high to low dose-rate transformation frequencies

Time after irradiation of cells irradiated at high dose-rate	Ratio for T.F. per viable cell ± standard error	Ratio for T.F. per cell at risk ± standard error
0 hours	1.9 ± 1.0	1.8 ± 1.4
5 hours	2.6 ± 1.2	2.8 ± 2.1
Average	2.3 ± 1.0	2.3 ± 1.7

Table shows the ratios calculated of the high to low dose-rate data for transformation frequencies (T.F.) after the frequencies were corrected for background transformation. The low dose-rate data is compared to each of the high dose-rate frequencies as well as to the average transformation frequency of the cells irradiated at high dose-rate.

Summary and Discussion

Reduction of the dose-rate at which C3H10T½ cells are exposed to 3 Gy X-rays from an exposure time of a few minutes to five hours greatly reduces the observed transformation frequency per surviving cell and per cell at risk. The fraction of cells surviving the irradiation increases at the lower dose-rate. When the cells exposed to the higher dose-rate are incubated for some time before assessing the transformation frequency no difference is found between seeding immediately after irradiation and five hours after irradiation, while an enhanced transformation frequency is noted when the transformation is assessed about an hour after irradiation.

The effect of the reduction of the dose-rate at which cells are exposed to radiation is discussed here solely with reference to low-LET radiation. Data on high-LET radiation has been outlined in chapter one. The lower transformation induced by X-rays at reduced dose-rates reported in this chapter has also been reported in previous publications for both X-rays and gamma rays (for example, Miller *et al.* 1978, 1979, Terzaghi *et al.* 1976, Han *et al.* 1979, Hill *et al.* 1984, Terasima *et al.* 1985, Watanabe *et al.* 1984, Balcer-Kubiczek *et al.* 1987, 1989). There is general agreement of reduced transformation with reduced dose-rate for doses greater than 1 Gy X-rays. However results vary for doses less than 1Gy X-rays with some data suggesting a reduced effectiveness with lower dose-rate (Terasima *et al.* 1985) while other data suggest an enhanced transformation with lower dose-rate (Miller *et al.* 1978, 1979). The decreased transformation frequency observed in the data presented here when the dose-rate is reduced cannot be explained solely by the increased survival of the irradiated cells, since the decreased transformation is still observed when one examines the transformation frequency per irradiated cell without taking account of cell survival.

The fidelity of repair of DNA damage is the major determinant of the dose-rate effect and it appears that repair of both potentially lethal and of potentially transforming damage occurs during irradiation at lower dose-rates (for example, Balcer-Kubiczek *et al.* 1989, Terasima *et al.* 1985, Watanabe *et al.* 1984). Balcer-Kubiczek *et al.* (1989) concluded from their studies that the repair time for potentially transforming damage is of the same general magnitude (one to four hours) as that for sublethal lesion repair (Elkind 1984) and chromosome break repair (Lloyd *et al.* 1984).

Appendices

Dose	Experiment	Plating Efficiency /%	Surviving Fraction	Total Cells Plated	Cells at Risk per Flask	Viable Cell Density x cm^{-2}	Number of Flasks	Number Cells at Risk	Flasks with Foci	Flasks without Foci	Mean Number of Foci per Flask	Transformation Frequency per Viable Cell x10000	Surface Area /m^2
Control	1	63 5	1 0	1293	821	4 69	30	24636	0	30	0 000 ± 0 000		0 53
	2	60 2	1 0	719	433	2 47	29	12559	1	28	0 035 ± 0 035		0 51
	3	41 5	1 0	895	371	2 12	59	21893	1	58	0 017 ± 0 017		1 03
	4	51 8	1 0	905	469	2 68	60	28122	2	58	0 034 ± 0 024		1 05
	TOTAL/AVERAGE		1 000			2 80	178	87209	4	174	0 023 ± 0 011	0 46 ± 0 23	3 12
	STANDARD ERROR		0 000										
	5 (0 hours)	28 0	1 0	891	249	1 54	28	6985	1	27	0 036 ± 0 036		0 45
	5 (5 hours)	28 0	1 0	900	252	1 44	34	8568	0	34	0 000 ± 0 000		0 60
	6 (0 hours)	54 0	1 0	900	486	2 78	28	13608	2	26	0 074 ± 0 051		0 49
	6 (5 hours)	55 8	1 0	925	516	2 95	14	7226	1	13	0 074 ± 0 073		
	TOTAL/AVERAGE		1 000			2 36	104	36388	4	100	0 039 ± 0 019	1 12 ± 0 56	1 54
	STANDARD ERROR		0 000										
High dose-rate (0 hours)	1	33	0 52	2904	506	3 12	90	45558	8	82	0 093 ± 0.032		1 46
	2	30	0 50	1227	187	1 15	50	9352	1	49	0 020 ± 0.020		0 81
	3	30	0 71	1413	297	1 84	119	35387	6	113	0 052 ± 0.021		1 93
	4	26	0 50	1388	181	1 03	119	21511	8	111	0 070 ± 0 024		2 08
	TOTAL/AVERAGE		0 560			1 78	378	111808	23	355	0 063 ± 0.013	2 12 ± 0 44	6.28
	STANDARD ERROR		0 051										
	5	7	0 26	1384	23	0 14	89	2211	16	73	0 198 ± 0 047		1 56
	6	26	0 48	1408	177	1 01	56	9901	10	46	0 197 ± 0.059		0 98
	TOTAL/AVERAGE					0 48	145	12111	26	119	0 198 ± 0 037	23 66 ± 4 42	2 54
	STANDARD ERROR												
High dose-rate (0 8 hours)	5	12	0 43	1384	71	0 44	39	2763	8	31	0 230 ± 0 077		0 63
	6	24	0 48	1407	162	1 00	83	13444	8	75	0 101 ± 0 035		1 34
	TOTAL/AVERAGE		0 460			0 82	122	16207	16	106	0 141 ± 0 034	10 58 ± 2 56	1 98
	STANDARD ERROR		0 040										
High dose-rate (1 4 hours)	5	18	0 63	1395	157	0 97	90	14153	21	69	0 266 ± 0 054		1 46
	TOTAL/AVERAGE		0.630			0 97	90	14153	21	69	0 266 ± 0 054	16 90 ± 3 46	1 46
	STANDARD ERROR		0 110										
High dose-rate (5 hours)	1	37	0 71	2891	759	4 68	73	55389	9	64	0 132 ± 0 0425		0 00 1 18
	2	32	0 60	1210	229	1 41	79	18070	3	76	0 039 ± 0 0221		1 28
	3	23	0 41	1407	131	0 81	119	15635	2	117	0 017 ± 0 0119		1 93
	4	25	0 52	1389	183	1 04	92	16814	14	78	0 165 ± 0 0424		1 61
	TOTAL/AVERAGE		0 561			1 77	363	105908	28	335	0 080 ± 0 015	2.75 ± 0 51	6.00
	STANDARD ERROR		0 065										
	5	13	0 48	1387	86	0.49	96	8283	18	78	0 208 ± 0 0465		1 69
	6	26	0 47	1408	173	0 99	57	9848	7	50	0 131 ± 0 0479		1 00
	TOTAL/AVERAGE					0 68	153	18131	25	128	0 178 ± 0 034	15 05 ± 2 88	2 68
	STANDARD ERROR												
Low dose-rate	1	36	0 69	2892	707	4 37	120	84893	12	108	0 105 ± 0 0296		1 94
	2	36	0 68	1197	289	1 78	115	33239	2	113	0 018 ± 0 0124		1 86
	3	33	0 57	1411	263	1 62	118	31045	2	116	0 017 ± 0 0120		1 91
	4	32	0 67	1399	298	1 71	120	35819	8	112	0 069 ± 0 0240		2 10 0 00
	TOTAL/AVERAGE		0 632			2 37	473	184996	24	449	0 052 ± 0 0105	1 33 ± 0 27	7 82
	STANDARD ERROR		0 026										

Appendix 4.1. Individual experiment data and transformation frequencies calculated per viable cell. High refers to the high dose-rate of 0.8 Gy per minute and low refers to the low dose-rate of 0.01 Gy per minute. The time given for each high dose-rate is the time after irradiation at which the transformation frequency was assessed.

Chapter five

Characterisation of C3H10T½ foci

Section 5.1. Focus isolation and classification

Section 5.2. Tumourigenicity tests

Section 5.3. Focus Reconstruction studies

Section 5.4. Growth *in vitro* studies

Section 5.5. Cytogenetic studies

Section 5.6. Discussion of transformation properties

Section 5.1.

Focus isolation and classification

List of tables and figures

The C3H10T½ assay is extensively used as a system to assess the carcinogenic properties of a variety of agents including chemicals and radiation. It is the loss of contact inhibition of the cells when they become transformed that makes the system particularly attractive. This loss of contact inhibition is observed as a focus of cells which grow in layers. Although the foci are easily distinguished against a monolayer of contact - inhibited cells, the decision as to what constitutes a positively transformed focus is not as easily resolved. Positively transformed *in vitro* is considered to be equivalent to tumourigenic *in vivo*, as one cannot truly apply the term tumourigenic to these foci until they have proved capable of producing tumours. The foci produced in the transformation assay were originally classified into three types, designated types I, II and III, by Reznikoff *et al.* in their publication in 1973. Type I foci, composed of tightly packed cells are not scored as malignantly transformed, since foci isolated by Reznikoff *et al.* failed to produce tumours in C3H mice. Type II foci show considerable piling-up of cells into virtually opaque multilayers with criss-crossing of cells not pronounced. The third category of focus, type III consists of multilayered criss-crossing arrays of densely stained fibroblastic cells. Type II and III are classified as malignantly transformed, with fifty percent of type II and eighty five percent of type III producing tumours in C3H mice, as reported by Reznikoff *et al.*

Not all foci in the C3H10T½ assay fall easily into one of these categories. A wide range of foci are usually observed with some or all of the above characteristics and classifying the foci as I, II or III often disguises borderline cases. These borderline foci can create significant differences in the data obtained in different laboratories, depending on the manner of scoring of the foci and the criteria deemed most important for the distinction between positively and negatively transformed foci. A catalogue illustrating the range of foci observed and the classification attributed to the foci has been prepared as part of the collaboration project described in chapter three (copy available from any of the authors).

The most definitive test of positively transformed cells *in vitro* is the ability of the cells to produce tumours *in vivo*. However it is not feasible to isolate and test all the transformed foci produced in a typical C3H10T½ assay. Several authors have isolated sample foci and tested the tumourigenicity (see section 5.2). In this thesis,

data on the isolation and examination of twenty seven X-ray induced foci, one spontaneous focus and sixty alpha-particle induced foci are presented. The isolation and classification of these foci are described in the following sections.

Focus isolation

Foci isolated, expanded and developed as individual cell lines for use in the majority of the work in this thesis were produced after irradiation with X-rays or alpha-particles. Parent C3H10T½ cells were exposed to 5 Gy X-rays or 1 Gy alpha-particles using the radiation sources as described in chapter two. A standard transformation assay was then set up (see chapter two) but the cultures were incubated for ten weeks instead of the standard six weeks adopted by many laboratories or the four weeks postconfluence incubation adopted by the European collaborative project (details in chapter three). The longer incubation period allowed the foci to grow to a size which made the isolation of the foci more feasible. Foci to be isolated, were photographed the day before isolation, while still in culture. Isolation of the focus cells was accomplished by scraping approximately half of the focus, using a cell scraper into a small volume of medium which was then aspirated into a separate tissue culture flask (containing growth medium). The cells were left to attach and grow and subsequently subcultured into larger tissue culture flasks for further growth to cell numbers which allowed frozen stocks of each focus to be established as described in chapter two. The remaining culture, from which the focus was originally isolated was stained with a giemsa stain (described in chapter two) and the focus classified using the criteria described below. The area of the focus remaining on the culture flask was measured using a grid divided into squares of nine square millimetres in area and the total area of the focus estimated using the same grid. The percentage focus remaining was then calculated. The alpha-particle induced foci were isolated in two batches, with a week between batches while the X-ray induced foci were isolated together from a single experiment.

Focus classification

The foci isolated were examined and categorised twice. The author examined

the stained remainder of the focus and classified it using the criteria of Reznikoff *et al.* (1973), but also including a new classification (type X) described below. Foci were again categorised as part of a collaborative project with four other laboratories. The project began in 1990 with laboratories in Berkeley and Harwell in the United Kingdom, Milan and Rome in Italy and Munich and Göttingen in Germany (details in chapter three) working together to standardise the C3H10T½ assay for use by collaborating laboratories examining the transforming effects of low doses of radiation. One especially important aim of the project was to standardise the criteria for categorisation of foci. The foci tabulated below were examined, using the stained remainder of the focus and the photographs taken prior to focus isolation, at meetings of the Berkeley, Milan, Rome, Munich and Göttingen laboratories (eight to ten people examined the foci). These laboratories have adopted the criteria of Reznikoff *et al.* with a few modifications. No distinction is made between type II and type III foci and the most important criteria for a positively transformed focus is the presence of criss-crossing cells. Thus heavily piled-up foci with even a few cells criss-crossing are regarded as positively transformed while a focus presenting piled-up cells only is deemed to be negatively transformed. A new category of focus has been identified in the process of this collaboration which is designated type X. Type X foci present as long, flowing, fibrous sheets of cells which may exhibit piling up of the cells along the strands but do not display criss-crossing of cells. This morphology is occasionally seen in conjunction with criss-crossing in a positively transformed focus.

Table 5.1.1 summarises the various categories into which the foci were placed. Tables 5.1.2 and 5.1.3 list the X-ray induced foci, spontaneous focus and alpha-particle induced foci with the corresponding classifications, details of the percentage focus stained and examined microscopically, as well as comments on the foci by the author. Figures one to six show photographs of the stained remainder of a number of the foci from different categories.

It is evident from tables 5.1.2 and 5.1.3 that the area of the X-ray induced foci ranged from 54mm^2 to 450mm^2 while that of the alpha-particle induced foci ranged from 18mm^2 to 639mm^2 with the majority of the foci having areas in the lower half of these ranges. The percentage focus remaining on the culture flask and stained was

up to ninety percent with most foci having below fifty percent stained. Thus in most cases over half of the focus area was removed to develop the cell line and less than half of the focus available to be examined by routine focus classification criteria. Comparison of the author's score for the foci with that of the European collaborative group reveals about fifty percent agreement for the X-ray induced foci and about seventy percent for the alpha-particle induced foci. In most cases of disagreement between the scores a higher proportion of foci were scored as (+) by the collaborative group and placed in other categories by the author. In a number of cases there were additional foci on the culture flasks to those foci isolated, and these foci mostly resembled the focus from which cells were isolated.

All of the isolated foci described below have been tested for the ability to induce tumours in C3H mice. A selection of foci from the various categories have been further tested for the growth characteristics, chromosome complement and ability to reconstruct foci, results of which are presented in the following sections of this chapter.

Table 5.1.1. Key to Focus Categorisation.

Focus Category	Description
+	Transformed focus, definite type II or III with criss-crossing arrays of cells
-	Not transformed
X	Flowing fibrous sheaths of cells, cells may be piled up but are not criss-crossed
X/+	Mixed characteristics of type (X) and (+) focus categories
Additional terms used to describe the foci	
Term	Meaning
I	Not transformed, focus of tightly packed cells
II	Transformed focus, several layers of cells, criss-crossing of cells not pronounced
III	Transformed focus, several layers of densely stained, criss-crossing arrays of cells
?	Focus difficult to score, category undecided

The table outlines the categories (+), (X/+), (X) and (-) used for scoring the foci listed in the following tables as well as additional terms used in the text to describe the foci. The terms I, II and III refer to the categories originally outlined by Reznikoff *et al.* (1973), while the categories (+), (X/+), (X) and (-) refer to the categories used for foci examined by the European collaborative group as described in the text.

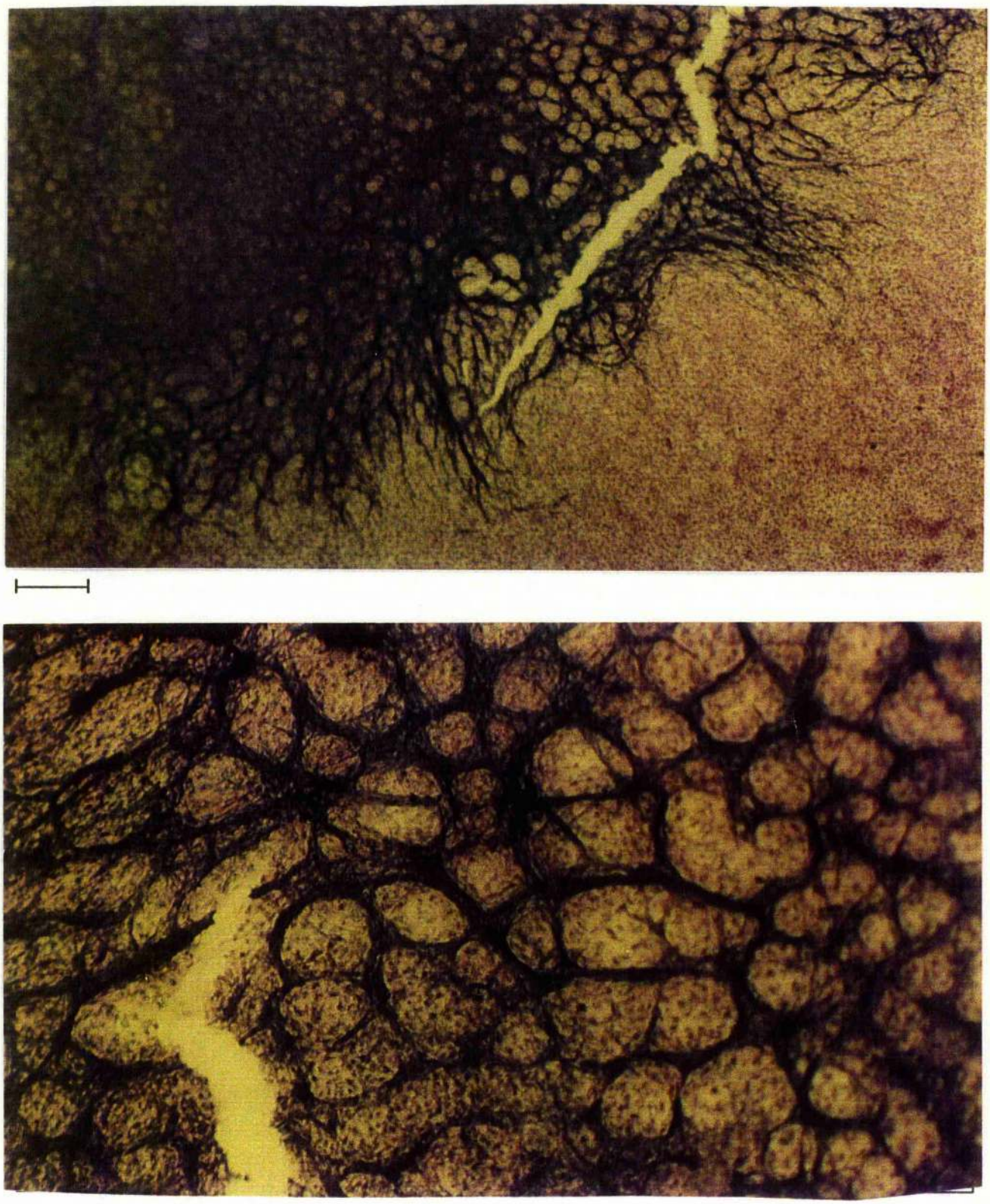


Figure one: Photographs of the X-ray induced focus X19. Lines under the photographs represent one millimetre as determined using photographs of a 1mm Objective micrometer (Nikon). The collaborative consensus score for this focus was (+).

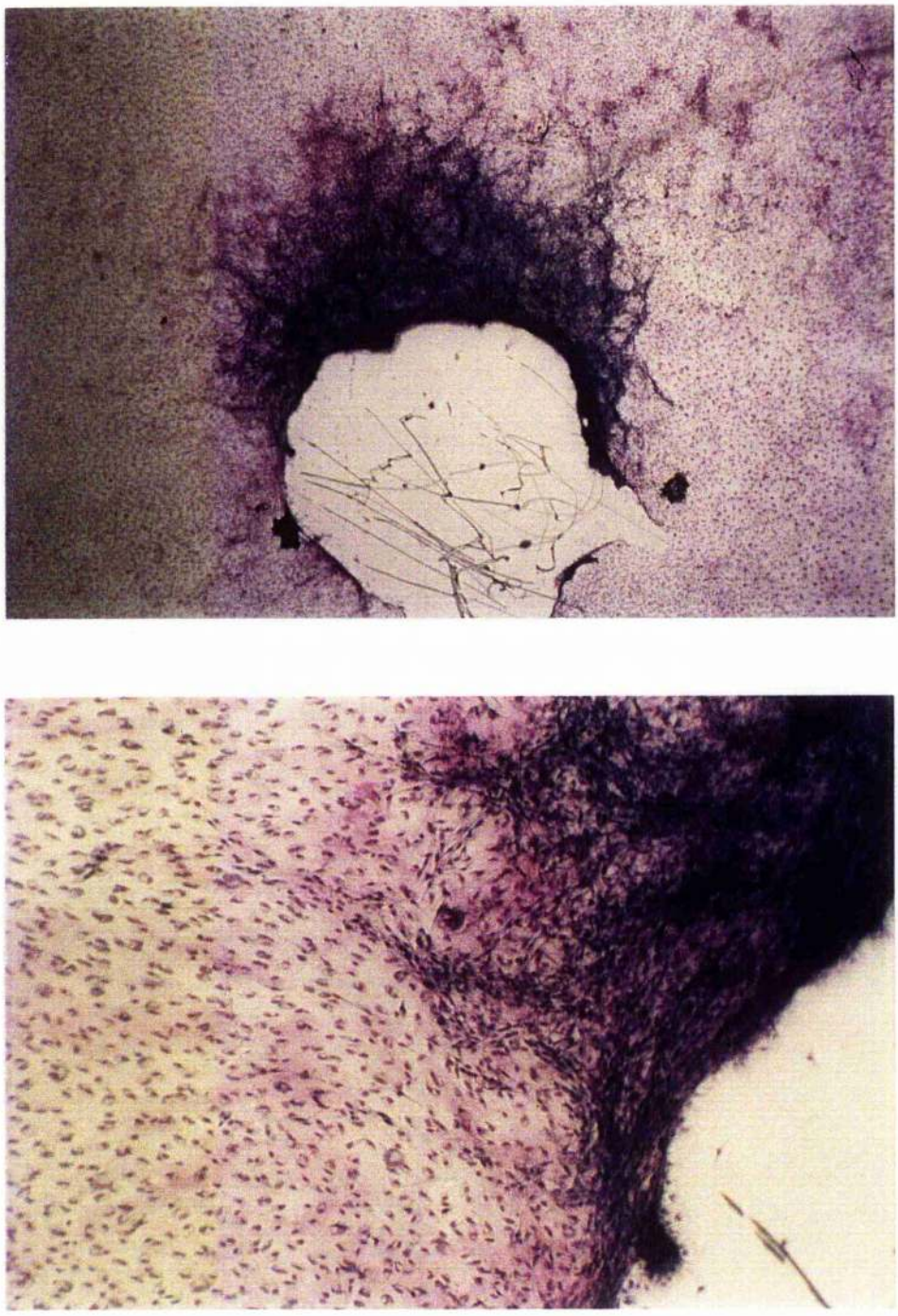


Figure two: Photographs of the alpha -particle induced focus α11. Lines under the photographs represent one millimetre as determined using photographs of a 1mm Objective micrometer (Nikon). The collaborative consensus score for this focus was (+).

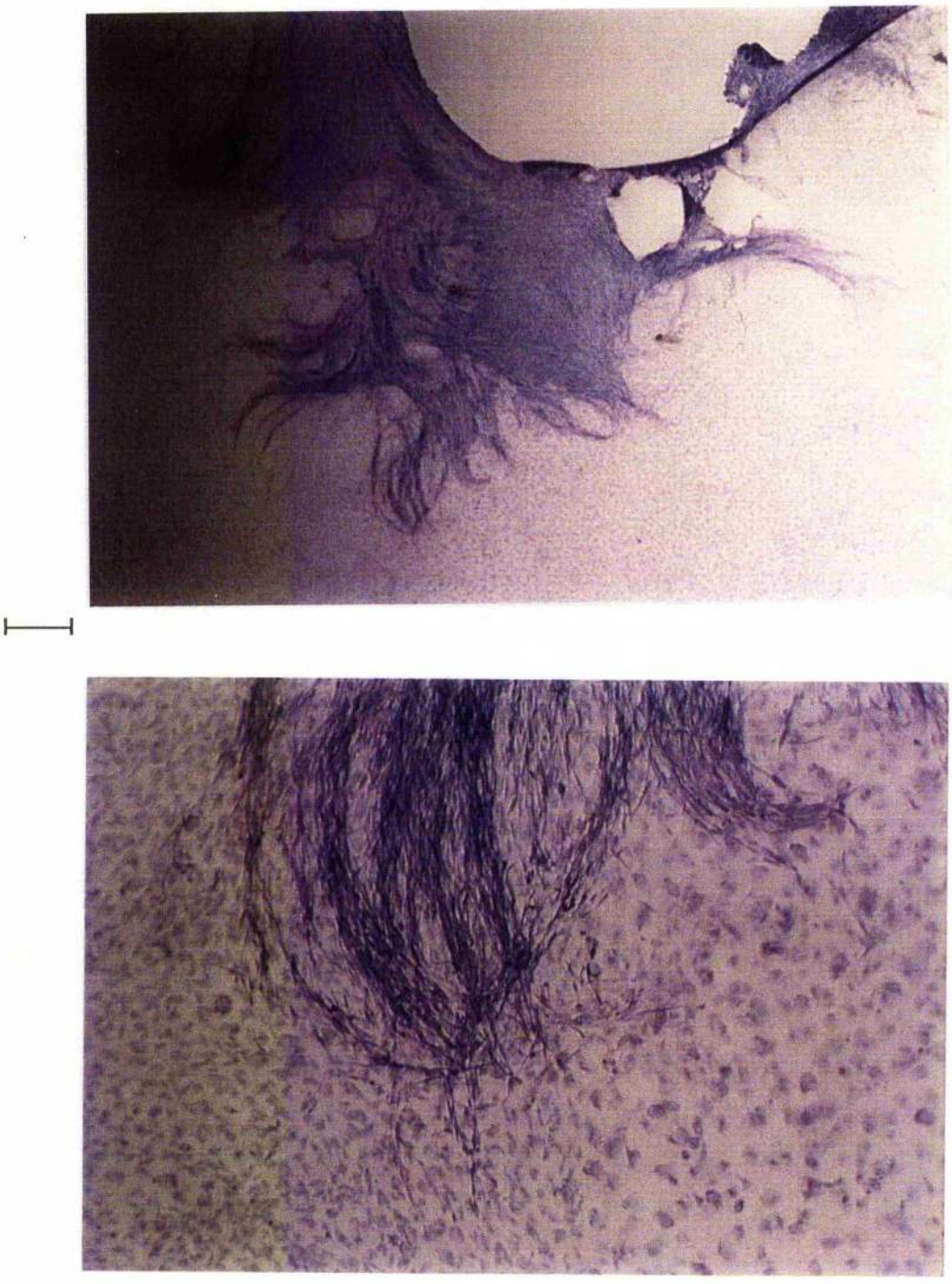


Figure three: Photographs of the X-ray induced focus X17. Lines under the photographs represent one millimetre as determined using photographs of a 1mm Objective micrometer (Nikon). The collaborative consensus score for this focus was (X/+).

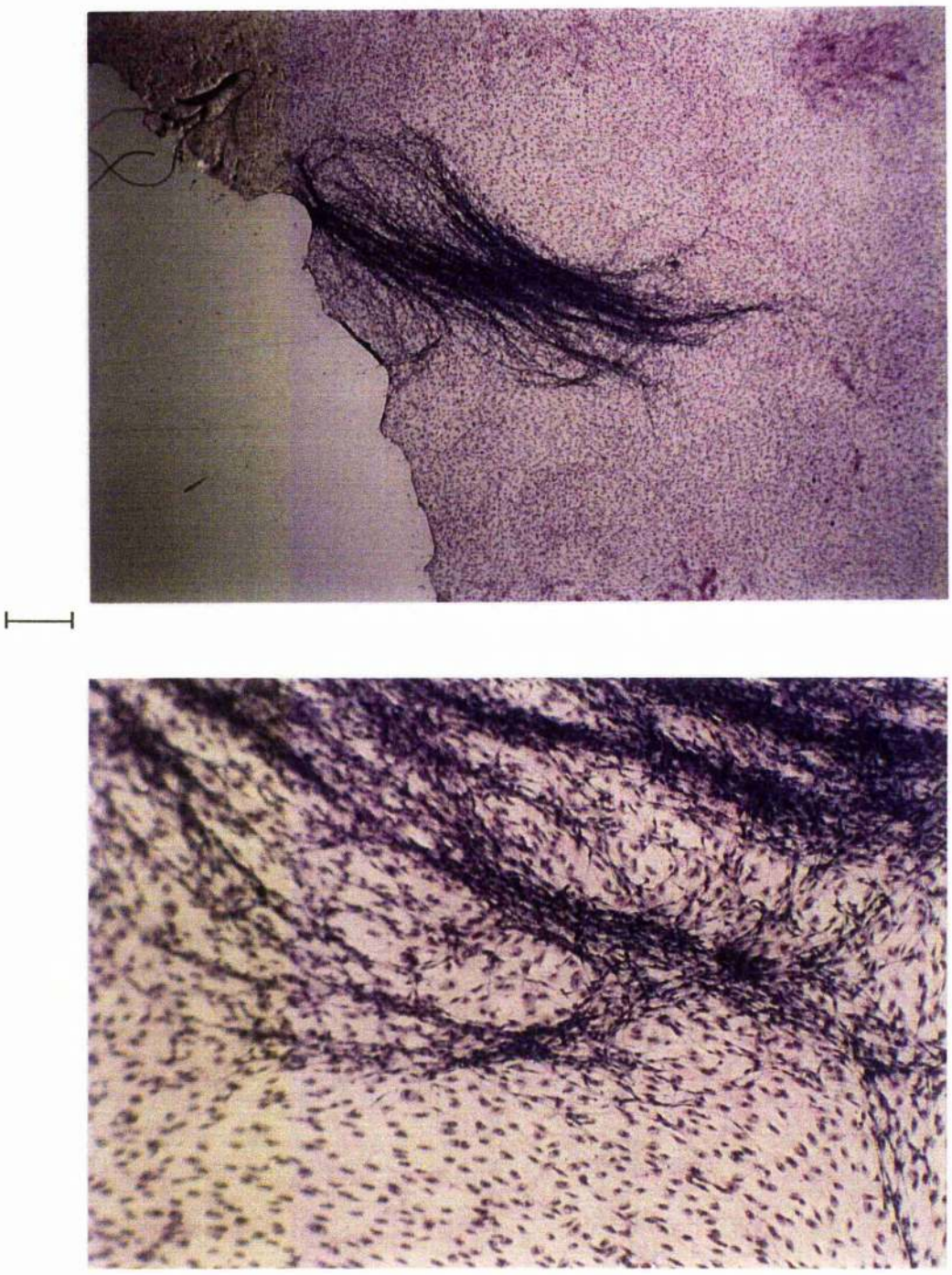


Figure four: Photographs of the X-ray induced focus X4. Lines under the photographs represent one millimetre as determined using photographs of a 1mm Objective micrometer (Nikon). The collaborative consensus score for this focus was (X/+).

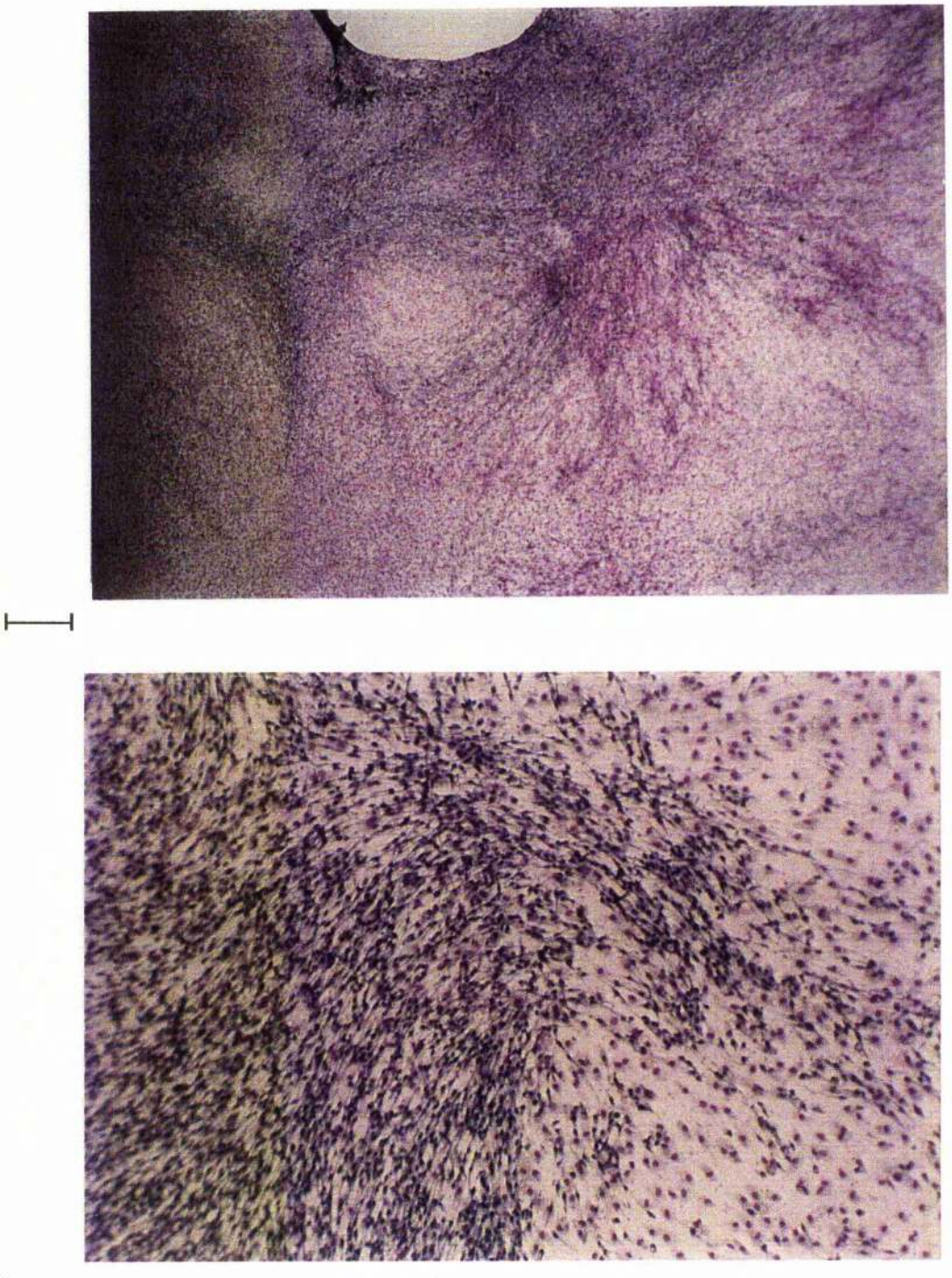


Figure five: Photographs of the X-ray induced focus X2. Lines under the photographs represent one millimetre as determined using photographs of a 1mm Objective micrometer (Nikon). The collaborative consensus score for this focus was (X).

Figure six: Photographs of the X-ray induced focus X6. Lines under the photographs represent one millimetre as determined using photographs of a 1mm Objective micrometer (Nikon). The collaborative consensus score for this focus was (-?).

Table 5.1.2. X-ray induced and spontaneous foci.

Focus label	Score		Focus remaining	Total focus area (mm^2)	Comments on foci
	Author	European			
X1	X	+	88%	225	X characteristics, some cells criss-crossing at the end of the strands of cells
X2	X	X	89%	162	Type I and X characteristics, another focus also on the culture dish (type II) with some of the focus missing, the resulting focus cell line may be a mixture of both foci
X3	-	+	83%	108	Characteristics of both types I and II
X4	X	X/+	7%	405	Very little focus left, some X strands remaining. Another focus (X and /or type II) on culture dish and several smaller satellite foci.
X5	?	+	0%	333	None of the focus remains
X6	II?	-?	12%	72	Small area remaining, type II
X7	?	+?	61%	279	Type I with some X characteristics, satellite foci also on culture dish
X8	II	+	67%	135	Dense type II focus
X9	-	+	57%	63	Focus with type I and II characteristics, another focus (type I) also on the culture dish
X10	?	+	0%	189	None of the focus remains, another focus on the culture dish (type II)
X11	X	X	4%	234	X characteristics, several satellite foci also on the culture dish (consisting of single layer streams of cells)
X12	X	X/+	73%	99	X characteristics, focus consists of single layer streams of cells

Focus label	Score		Focus remaining	Total focus area (mm^2)	Comments on foci
	Author	European			
X13	II/X?	+	61%	162	Traits of type II and type X, another similar focus also on the culture dish
X14	I	-?	50%	144	Type I, two other foci (type X and type I) also on culture dish
X15	II/III	+	4%	216	Border focus between type II and III, another focus (type I) also on the dish
X16	II	+?	14%	450	Type II, several similar satellite foci also present
X17	X	X/+	58%	108	X traits, several layers of streams of cells, some criss-crossing cells at the end of the streams
X18	I	-	59%	153	Type I, five other similar foci also present
X19	III	+	62%	234	Type III with a significant number of criss-crossing cells
X20	I/X	X/+	82%	153	Combination of type I and X foci, single layer streams of cells
X21	?	-?	0%	135	None of the focus remains
X22	+	+	4%	252	Type III, very little of the focus remains, several other foci also present (type I)
X23	II	+	59%	198	Traits of types I and II, some criss-crossing cells, six other similar foci also present
X24	?	+	0%	144	None of the focus remains
X25	+	-?	34%	54	Traits of I and II foci
X26	+	+	0%	135	None of the focus remains
X27	+	+	0%	198	None of the focus remains

Focus label	Score		Focus remaining	Total focus area (mm^2)	Comments on foci
	Author	European			
STR	+	+	64%	297	Dense type III, five other type III foci also present

Foci isolated following X-ray treatment of C3H10T½ cells are listed in this table (denoted with an X followed by a number). The STR focus is a spontaneous focus. Also listed is the author's classification of the focus using the criteria of Reznikoff *et al.* (1973), the classification of the foci by a collaboration of a number of laboratories, as outlined in the text and the author's comments on the appearance of the stained foci with details of any other foci also present on the surrounding monolayer. The classifications are those described in table 5.1.1. When none of the focus remained on the culture dish the focus classification was decided by examination of the photographs taken prior to focus isolation.

Table 5.1.3. Alpha-particle induced foci.

Focus label	Score		Focus remaining	Total focus area (mm^2)	Comments on foci
	Author	European			
α1	-	+	14%	63	Type I, very little focus left
α2	X	X/+	67%	108	Type X and I characteristics
α3	?	+	0%	81	None of the focus remains
α4	-	-	18%	99	Type I
α5	X	X/+	40%	270	Type X, several other type X foci also present
α6	-	+?	60%	225	Type II, some cells criss-crossing, two other foci (type II and type III) also present on the dish
α7	-	-	75%	36	Type I, several other type I foci also present
α8	+?	+?	69%	144	Combination of type I and type II, some criss-crossing cells
α9	-	-	79%	216	Type I, several other foci (X and/or III, two type I foci) also present
α10	?	+	0%	288	Type III, several type I foci on the culture dish
α11	+	+	60%	45	Type II, some criss-crossing cells

Focus label	Score		Focus remaining	Total focus area (mm^2)	Comments on foci
	Author	European			
α12	+	+	33%	207	Type III
α13	+	X/+	17%	54	Type III and/or X, single, dense streams of cells crossing each other, close to a type I focus, other foci on the culture dish are a similar X/III focus, a type X focus and a type I focus
α14	-?	-?	33%	27	Type I
α15	?	+	0%	342	None of the focus remains, four other foci are also present (one type II, three X/III foci)
α16	?	+	1%	639	Type III, three other small type III foci also present
α17	-	-?	0%	108	Type I and type II characteristics
α18	+	+	40%	45	Type III and/or X, single, dense streams of cells crossing each other
α19	+	+	44%	135	Type II, criss-crossing cells, two type II and two type I foci are also present
α20	+?	+?	0%	18	None of the focus remains, another focus (type III) also present
α21	?	-?	0%	108	None of the focus remains
α22	?	X/+	0%	135	None of the focus remains
α23	-	-	50%	108	Type I
α24	?	-	0%	81	Type I, several other type I foci also present
α25	X/+	+	17%	54	Type III and/or X, single, dense streams of cells crossing each other, another similar focus and four type I foci are also present

Focus label	Score		Focus remaining	Total focus area (mm^2)	Comments on foci
	Author	European			
α26	+?	+	0%	99	None of the focus remains, several other foci also present, combination of type II and type X foci
α27	-?	+	50%	144	Type II focus, some cells criss-crossing
α28	-	+	56%	81	Type II focus, criss-crossing of cells not pronounced, two type I foci present
α29	+	+	0%	351	None of the focus remains, six other foci present, combination of type I and type X foci
α30	+	+	57%	63	Type III, patchy focus
α31	+?	X/+	57%	63	Type X, several other type X foci also present
α32	+	+?	31%	378	Type II, some criss-crossing cells
α33	+	+	0%	108	None of the focus remains
α34	-?	-	16%	621	Type II
α35	X/+	+	38%	117	Combination of type III and X characteristics, another similar focus also present
α36	+	-?	0%	216	None of the focus remains, very small type II focus nearby
α37	+	+?	57%	252	Combination of type II and X characteristics, five other similar foci also present
α38	-	-	45%	99	Type I, four type I and one type II foci also present
α39	-	-	50%	36	Type I

Focus label	Score		Focus remaining	Total focus area (mm^2)	Comments on foci
	Author	European			
α40	+	+	14%	63	Type III and/or X, single, dense streams of cells crossing each other
α41	-	-	0%	108	None of the focus remains, large type I focus also present
α42	-	-?	0%	135	None of the focus remains, two type II foci also present on the culture dish, no cells criss-crossing
α43	+	-	50%	90	Type II, strands of cells which appear to cross
α44	-	-?	33%	81	Type I, another type I focus also present
α45	+	+?	40%	225	Combination of type X and II characteristics, some cells criss-crossing, two other foci (type I) also present
α46	-?	+?	40%	180	Combination of type II and III, centre of the focus missing, monolayer underneath the focus visible, six other similar foci also present
α47	+	+?	0%	117	Type I focus with some cells which appear to cross each other, several other similar foci
α48	+?	-	81%	234	Type I
α49	+	+?	3%	315	Type II, nine other type II foci also present
α50	+	+?	52%	189	Type I focus with some cells which appear to cross each other, several other type I foci
α51	X?/+	X?	0%	108	None of the focus remains, several X type foci present
α52	-	-	47%	171	Type II focus, two type II foci and one X and/or III focus also present
α53	-	+?	50%	270	Type II focus, some criss-crossing cells, two other similar foci also present

Focus label	Score		Focus remaining	Total focus area (mm^2)	Comments on foci
	Author	European			
α54	+	+	44%	162	Type III and/or X, single, dense streams of cells crossing each other, type I focus also present
α55	-	-	50%	360	Type I focus, several other foci (type I and/or II) also present
α56	+	+	55%	198	Type II focus, some criss-crossing cells in one area
α57	+?	+?	50%	72	Type II focus, some criss-crossing cells in one area, type I focus also present
α58	+	+	25%	36	Type III, another type III focus also present
α59	+	+?	18%	252	Type III
α60	+	+	50%	36	Type III, small type X focus also present on the culture dish

Foci isolated following alpha-particle irradiation of C3H10T½ cells are listed in this table (denoted with an α followed by a number). Also listed is the author's classification of the focus using the criteria of Reznikoff *et al.* (1973), the classification of the foci by a collaboration of a number of laboratories, as outlined in the text and the author's comments on the appearance of the stained foci and details of any other foci also present on the surrounding monolayer. The classifications are those described in table 5.1.1. When none of the focus remained on the culture dish the focus classification was decided by examination of the photographs taken prior to focus isolation.

Section 5.2.

Results of Tumourigenicity Studies

List of tables and figures

Figure 5.2.10 (a, b, c) Relationship of the tumour incidence to the time in culture of cells prior to tumourigenicity testing of (a) radiation - induced foci (combined data of X-rays and alpha - particles), (b) X-ray induced foci and (c) alpha - particle induced foci

The ultimate and definitive demonstration of the malignant nature of transformed cells as identified by *in vitro* assays is the ability of these cells to produce tumours in immunosuppressed animals. All the cell lines derived from C3H10T½ foci as described in section 5.1. have been tested for the capability to induce tumours in immunosuppressed C3H mice. The tumourigenicity studies were carried out at the University of St. Andrews, Scotland and the details of the procedure are provided in chapter two.

Tumourigenicity data of other authors

Table 5.2.1. outlines a number of publications by other authors on the tumourigenicity of C3H10T½ cells transformed by a variety of agents. The foci from which the cell lines were derived and tested in all these publications were classified into type I, II or III foci, using the criteria of Reznikoff *et al.* (1973). Most of the studies have used chemicals to induce the foci, with over a hundred foci induced by chemicals tested for tumourigenicity while data presented for X-ray induced foci comprised a total of thirty foci examined for tumourigenicity in two publications (Otsu *et al.* (1983), Paquette and Little (1992)), and there were no reports found for alpha-particle induced foci. The data presented by Otsu *et al.* and Paquette and Little illustrate the scale of differences obtained in many laboratories where similar numbers of foci (type III) have been examined for tumourigenicity but where considerable difference in tumourigenicity has been found: - ninety-three percent tumourigenic (Otsu *et al.*) versus forty-five percent (Paquette and Little). These findings also differ from those first reported by Reznikoff *et al.* in the original C3H10T½ publication in 1973 where the tumourigenicity for type III foci was reported as eighty percent. One of the many variables in the C3H10T½ transformation assay, as described in section 5.1., is the subjective nature of what are considered to be the important criteria for positively transformed foci. This is especially so since no two foci appear the same, and there are a wide range of foci which are borderline between the types I, II and III classifications (as described by Reznikoff *et al.*). Since the calculation of transformation frequencies induced in this system by a variety of carcinogens relies on the identification of transformed foci, it is important to verify the relationship of

transformed foci identified *in vitro* to tumourigenicity *in vivo*. The study in this thesis presents data on the tumourigenicity of twenty-seven X-ray induced foci, sixty alpha-particle induced foci and one spontaneous focus. The majority of previous studies used a smaller sample of foci mostly considering only type II or III foci which are regarded as positively transformed. Furthermore Smith *et al.* (1993) found the tumourigenicity of the foci they isolated differed depending on the chemical used to induce the focus. The question arises as to whether foci induced by alpha - particles are more or less tumourigenic than the same classification of focus induced by X-rays and also whether such a difference exists between chemicals and radiation.

Table 5.2.1. Tumourigenicity data of other authors.

Publications on tumourigenicity of carcinogen-induced C3H10T½ focus cell lines (Fraction and percentage tumourigenic foci).				
Authors	Mice inoculated	Type II foci	Type III foci	Agent used to induce foci
Reznikoff *et al.* 1973	syngeneic C3H, 5-7 weeks old	3/6 (50%)	5/6 (83%)	MCA / DMBA
Otsu *et al.* 1983	male C3H/He, 3 weeks old	1/5 (20%)	13/14 (93%)	X-rays
		1/1 (100%)		spontaneous
Paquette & Little 1992	syngeneic C3H/HeNCr1BR		5/11 (45%)	X-rays
			4/6 (67%)	recloned foci from second X-ray dose
Male *et al.* 1987	female C3H, 5 weeks old	0/1 (0%)	2/2 (100%)	MCA + TPA
Chen & Herschmann 1988	athymic BALB/c, 4-6 weeks old	0/4 (0%) (Type II or III not specified)		UV + TPA
		1/1 (100%) (Type II or III not specified)		MCA
Smith *et al.* 1993	nude mice	3/4 (75%)	5/6 (83%)	Spontaneous
		7/9 (78%)	5/8 (63%)	MNNG
		4/17 (24%)	3/12 (25%)	BPDE
		2/14 (14%)	0/5 (0%)	MCA
		16/44 (36%)	13/31 (42%)	Total from Spontaneous, NG, BP, MCA

Table lists publications outlining details of the tumourigenicity testing of a number of cell lines developed by a number of authors from type II and type III foci induced by a variety of treatments. The fraction (and percentage) of tumourigenic foci is presented. (MCA (3-methylcholanthrene), DMBA (7,12-dimethylbenz(a)anthracene), TPA (12-O-tetradecanoyl phorbol acetate), UV (ultra-violet radiation), MNNG (N-methyl-N'-nitro-N-nitrosoguanidine), BPDE (benzo(a)pyrene diol epoxide)). Reznikoff et al. (1973) also tested four cell lines derived from type I foci, all of which were non-tumourigenic.

Tumour incidence

Table 5.2.2. lists the foci in the categories agreed by the European collaboration group as well as by the author alone (descriptions presented in tables 5.1.2. and 5.1.3. in section 5.1.) with the number of foci in each category which were tumourigenic. Tumourigenicity has been classified in three ways. Fully tumourigenic foci produced tumours in all mice inoculated, while partially tumourigenic foei produced tumours in at least one mouse inoculated and non-tumourigenic foci produced no tumours in any mice inoculated. These terms are used in the following tables and graphs, however the terms are limited to the conditions under which the foci were tested, for example, fully tumourigenic foci may have produced six tumours in the six mice tested but if ten mice were tested it may have only produced nine tumours. Similarly non tumourigenic foci may have produced tumours if more mice were innoculated. Table 5.2.3. illustrates the number of tumourigenic foci categorised using the focus categories described by Reznikoff *et al.* (1973). The foci were categorised in two ways. All foci contained a mixture of characteristics and were first classified according to "critical" characteristics in decreasing order of importance type III, II, X, I (routine method of focus classification). The second method of classification was to categorise the foci in all the focus categories they showed characteristics of, thus a focus described as displaying type II and type X characteristics was classified in both categories. Tables 5.2.4. (X-ray induced foci and spontaneous focus) and 5.2.5. (alpha-particle induced foci) present individual focus data including the individual tumour incidence, focus area and percentage focus isolated to develop the cell line (details from table 5.1.3. and 5.1.4. in section 5.1.) as well as the time scale of the first and last tumour appearance for each tumourigenic focus.

Table 5.2.2. Tumourigenicity results (Author versus European collaborative classification of foci).

Focus Classification by author or collaboration group	(+) / (+?)		(X/+) / (X/+?)		(X) / (X?)		(-) /(-?)		(?)	
X-ray induced foci										
A= Author's classification, C= European Collaboration classification										
	C	**A**	**C**	**A**	**C**	**A**	**C**	**A**	**C**	**A**
Number of foci classified	16	6	4	0	2	8	5	8	0	5
Number of fully tumourigenic foci	9	3	2	0	1	4	2	3	0	4
Number of partially tumourigenic foci	5	2	1	0	1	3	2	3	0	1
Number of non tumourigenic foci	2	1	1	0	0	1	1	2	0	0
α-particle induced foci										
	C	**A**	**C**	**A**	**C**	**A**	**C**	**A**	**C**	**A**
Number of foci tested	35	28	5	3	1	2	19	20	0	7
Number of fully tumourigenic foci	4	2	2	0	0	2	2	3	0	1
Number of partially tumourigenic foci	10	8	0	0	0	0	5	5	0	2
Number of non tumourigenic foci	21	18	3	3	1	0	12	12	0	4
Spontaneous focus										
Number of foci tested	1	1	0	0	0	0	0	0	0	0
Number of fully tumourigenic foci	1	1	0	0	0	0	0	0	0	0

Table shows the total number of foci in each category (as decided by the author alone (column (A)) or by the European collaboration group (column (C)) tested for tumourigenicity and the number of these which were fully, partially or non-tumourigenic. Fully tumourigenic foci produced tumours in all mice injected with the focus cells and partially tumourigenic foci produced a tumour in at least one mouse injected. The category (?) represents foci which could not easily be classified into any of the other categories. The author's classification of the foci relied on examination solely of the focus area stained while the collaboration group examined the stained focus and the photographs taken of the foci prior to isolation of focus cells. These data are presented for individual foci in tables 5.2.4. and 5.2.5.

Table 5.2.3. Tumourigenicity of foci categorised according to the critical focus characteristics and all focus characteristics.

Sample	Classification criteria		Type I	Type II	Type III	Type X	Category undecided
X-ray induced foci	"critical" focus characteristics	Number of foci	2	7	6	6	6
		Number of tumourigenic foci	2	6	4	5	6
		Percentage tumourigenicity	100	86	67	83	100
	combination of characteristics included	Number of foci	9	9	6	9	6
		Number of tumourigenic foci	8	6	4	8	6
		Percentage tumourigenicity	89	67	67	88	100
α-particle induced foci	"critical" focus characteristics	Number of foci	15	6	23	3	11
		Number of tumourigenic foci	3	2	9	2	6
		Percentage tumourigenicity	20	33	39	67	55
	combination of characteristics included	Number of foci	17	17	24	10	11
		Number of tumourigenic foci	4	8	10	3	6
		Percentage tumourigenicity	24	47	42	30	55

Foci rarely display characteristics of only one focus category. This table presents the tumourigenicity data of the foci when they were categorised (using the classifications of Reznikoff *et al.* (1973) and including type X) according to the critical characteristics as well as when foci were categorised to include all characteristics displayed. The critical criteria classification in decreasing order of importance was type III, type II, type X and type I. Foci with a mixture of characteristics (as outlined in table 5.1.1, chapter 5.1.) for example, from type II and type X were included in categories type II and X, in the above table but the critical classification was type II. Where none of the focus remained, the category of focus could not be decided. The classifications were based on the author's examination of the foci.

Table 5.2.4. X-ray induced and spontaneous foci details and tumourigenicity results

Focus				Number of mice		Tumour appearances	
Label	Type	Area (mm^2)	% area isolated	injected	with tumours	First	Last
X1	(+)	225	12	4	4	8	18
X3	(+)	108	17	4	4	3	6
X5	(+)	333	100	6	6	3	7
X7	(+?)	279	39	5	5	1	4
X8	(+)	135	33	6	1	35	
X9	(+)	63	43	3	1	25	
X9 (repeat)				4	0		
X10	(+)	189	100	5	5	1	4
X13	(+)	162	39	5	3	4	
X13 (repeat)				4	4	2	5
X15	(+)	216	96	4	0		
X16	(+?)	450	86	5	5	6	7
X19	(+)	234	38	5	5	3	5
X22	(+)	252	96	4	4	7	29
X22 (repeat)				3	0		
X23	(+)	198	41	4	0		
X23 (repeat)				4	0		
X24	(+)	144	100	5	5	7	19
X26	(+)	135	100	3	1	14	
X26				4	4	3	10
X27	(+)	198	100	5	5	1	3
X4	(X/+)	405	93	5	4	3	4
X12	(X/+)	99	27	4	0		
X17	(X/+)	108	42	5	5	7	14
X20	(X/+)	153	18	7	7	2	12
X2	(X)	162	11	3	3	12	18
X11	(X)	234	96	3	1	12	
X11 (repeat)				3	0		
X6	(-?)	72	88	4	0		

Focus				Number of mice		Tumour appearances	
Label	Type	Area (mm^2)	% area isolated	injected	with tumours	First	Last
X14	(-?)	144	50	6	6	6	8
X14 (repeat)				4	0		
X18	(-)	153	41	5	5	8	9
X18 (repeat)				4	4	5	11
X21	(-?)	135	100	5	4	4	5
X25	(-?)	54	66	4	4	4	7
STR	(+)	297	36	5	5	4	7

Table presents data for the X-ray induced foci and the spontaneous focus (STR), in order of classification (+), (X/+), (X), (-) (European collaborative classification) on the tumourigenicity results, tumour latency periods as well as the estimated size of the focus from which the cell line was derived and the fraction of the focus which was isolated to produce the cell line. Data on two tumour incidences for one focus indicate repeat tests.

Table 5.2.5. Alpha-particle induced foci details and tumourigenicity results.

Focus				Number of mice		Tumour appearances	
Label	Type	Area (mm^2)	% area isolated	injected	with tumours	First	Last
α1	(+)	63	86	4	0		
α3	(+)	81	100	4	0		
α6	(+?)	225	40	4	0		
α8	(+?)	144	31	3	0		
α10	(+)	288	100	2	2	7	18
α10 (repeat)				4	4	1	31
α11	(+)	45	40	4	4	3	11
α12	(+)	207	67	4	0		
α15	(+)	54	83	4	0		
α16	(+)	639	99	6	5	2	6
α18	(+)	45	60	4	0		
α19	(+)	135	56	4	4	6	12
α20	(+?)	18	100	4	2	20	31
α25	(+)	54	83	3	0		
α26	(+)	99	100	4	0		
α27	(+)	144	50	4	0		
α28	(+)	81	44	4	1	22	
α29	(+)	351	100	4	3	5	8
α30	(+)	63	43	4	0		
α32	(+?)	378	31	5	3	9	16
α33	(+)	108	100	4	0		
α35	(+)	117	62	4	0		
α37	(+?)	252	43	4	0		
α40	(+)	63	86	4	0		
α46	(+?)	180	60	4	4	6	9
α47	(+?)	117	100	4	0		
α49	(+?)	315	97	4	3	2	6
α50	(+?)	189	48	5	0		
α53	(+?)	270	50	4	3	9	14
α54	(+)	162	56	4	2	39	41
α56	(+)	198	45	4	3	6	21
α57	(+?)	72	50	4	0		

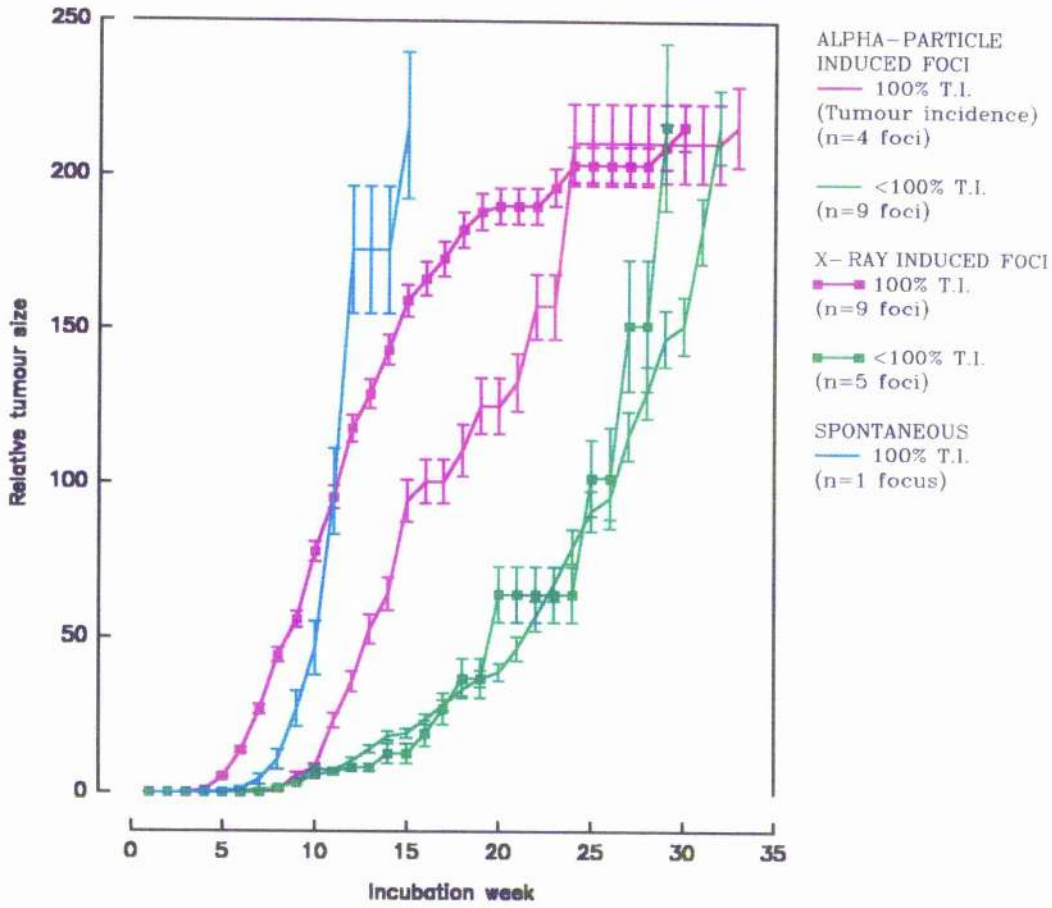
Focus				Number of mice		Tumour appearances	
Label	Type	Area (mm^2)	% area isolated	injected	with tumours	First	Last
α58	(+)	36	75	4	0		
α59	(+?)	252	82	4	0		
α60	(+)	36	50	4	1	27	
α2	(X/+)	108	33	4	4	7	16
α5	(X/+)	270	60	4	4	5	7
α13	(X/+)	54	83	4	0		
α31	(X/+)	63	43	3	0		
α51	(X)	108	100	4	0		
α4	(-)	99	82	4	4	7	27
α7	(-)	36	25	3	0		
α9	(-)	216	21	4	2	10	21
α14	(-?)	27	67	4	0		
α17	(-?)	108	100	5	0		
α21	(-?)	108	100	3	1	15	
α23	(-)	108	50	2	0		
α24	(-)	81	100	3	0		
α34	(-)	621	84	4	0		
α36	(-?)	216	100	4	3	9	10
α38	(-)	99	55	4	0		
α39	(-)	36	50	3	0		
α41	(-)	108	100	4	3	6	7
α42	(-?)	135	100	4	4	9	19
α43	(-)	90	50	4	0		
α44	(-?)	81	67	4	0		
α48	(-)	234	19	4	0		
α52	(-)	171	53	4	0		
α55	(-)	360	50	4	3	9	11

Table presents data for the α-particle induced foci, in order of classification (+), (X/+), (X), (-) (European collaborative classification) on the tumourigenicity results, tumour latency periods as well as the estimated size of the focus from which the cell line was derived and the fraction of the focus which was isolated to produce the cell line. Data on two tumour incidences for one focus indicate repeat tests.

The tumour studies were carried out at the University of St. Andrews where the mice were examined weekly for a maximum of thirty-five weeks and the sizes of any tumours recorded on a scale of one to six, approximating to tumour diameters of two to twelve millimetres. Once a tumour size six was reached the tumour was excised. The following figures (5.2.1. to 5.2.6.) present data on the induction and growth of tumours, comparing partially tumourigenic and fully tumourigenic foci, X-ray versus alpha-particle induced foci, and radiation-induced (combined data of alpha-particle and X-ray induced foci) versus spontaneous focus. The relative tumour size plotted was calculated proportional to tumour volume (r^3) assuming the tumour sizes one to six correspond to tumour radii (r) of one to six millimetres. Figures 5.2.1 (a, b, c, d) present the data for growth rate of tumours induced by the various categories of foci. Data on the totally, fully and partially tumourigenic radiation - induced foci (combined data of X-rays and alpha - particles) is presented in figures 5.2.2. and 5.2.3., and these data separated into radiation type are presented in figures 5.2.4. to 5.2.6. These data are also presented in tabular form in appendices one to three. When a tumour was excised, the tumour size six (corresponding to a tumour radius of six millimetres) was kept in the calculations to estimate what the average tumour size would have been for each week after the focus cells were injected into the C3H mice. The calculations for the tumour growth for the partially tumourigenic foci included only the tumour bearing mice. The remainder of the figures present data on the time of detection of the first and last tumours (5.2.7.) and the relationship of the tumour incidence to the focus area (5.2.8. to 5.2.9.) and to the time the cells were in culture before tumourigenicity testing was done (5.2.10.)

Tumour growth curves

Figure 5.2.1 (a) illustrates that the growth of the tumours induced by the partially tumourigenic (+) foci was slower with a more gradual increase in tumour size than that for tumours induced by fully tumourigenic (+) foci for both alpha-particle and X-ray induced foci. Fully tumourigenic X-ray induced (+) foci produced tumours sooner than the corresponding alpha-particle induced foci, but the growth curves are similar. There is no notable difference between the growth curves for the partially



(+) foci

Figure 5.2.1 (a). Growth of tumours induced by radiation - induced C3H10T½ focus cells.

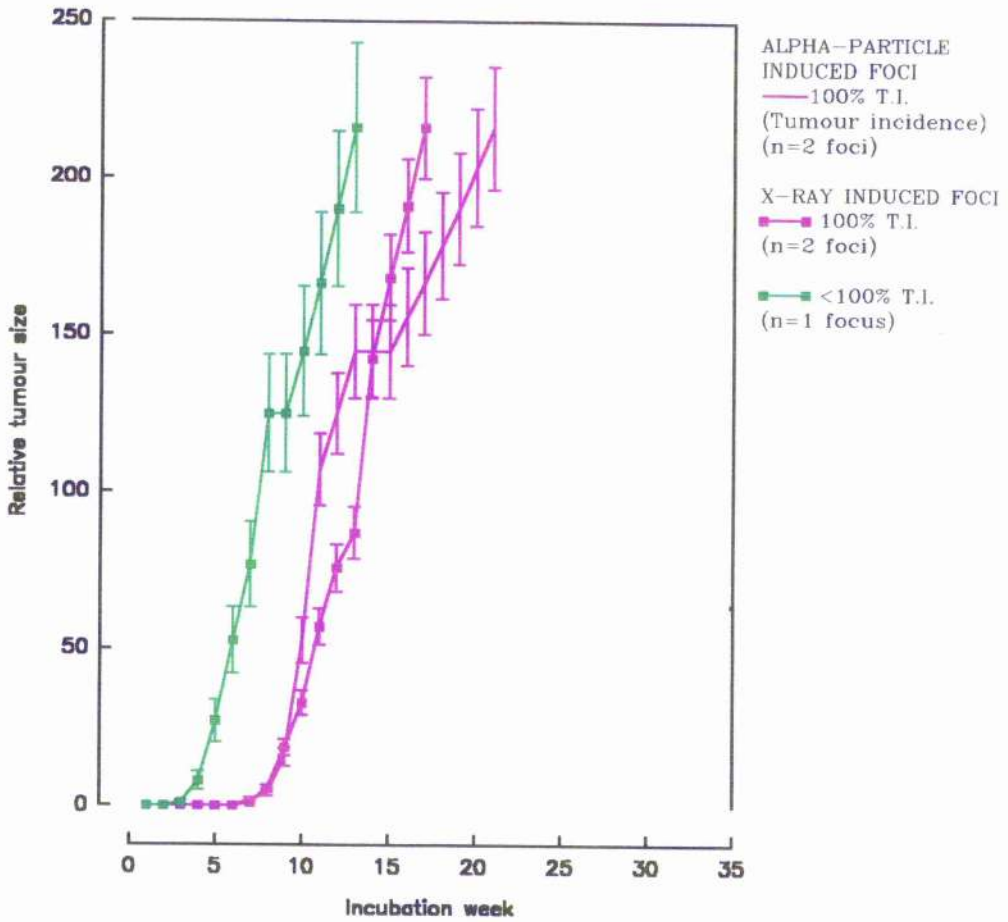
tumourigenic X-ray and alpha-particle induced (+) foci. Tumours induced by the spontaneous (+) focus grew rapidly, with all tumours apparent by fourteen weeks after injection of the focus cells into the mice, however only one focus was available for testing.

Figure 5.2.1 (b) presents data on the induction and growth of tumours by the (X/+) foci, a category where only a small number of foci were examined. There was no notable difference between the fully tumourigenic alpha-particle and X-ray induced (X/+) foci, especially for the smaller tumour sizes. No data were available for a partially tumourigenic alpha-particle induced (X/+) focus. The partially tumourigenic X-ray induced focus produced tumours sooner than the fully tumourigenic.

Data on the growth of tumours produced by (X) foci are presented in figure 5.2.1 (c). There were only a small number of tumourigenic (X) foci, none of which were induced by alpha-particles. Foci produced tumours twelve weeks after injection of the focus cells and the tumours produced by the partially tumourigenic focus grew quicker than tumours produced by the fully tumourigenic focus.

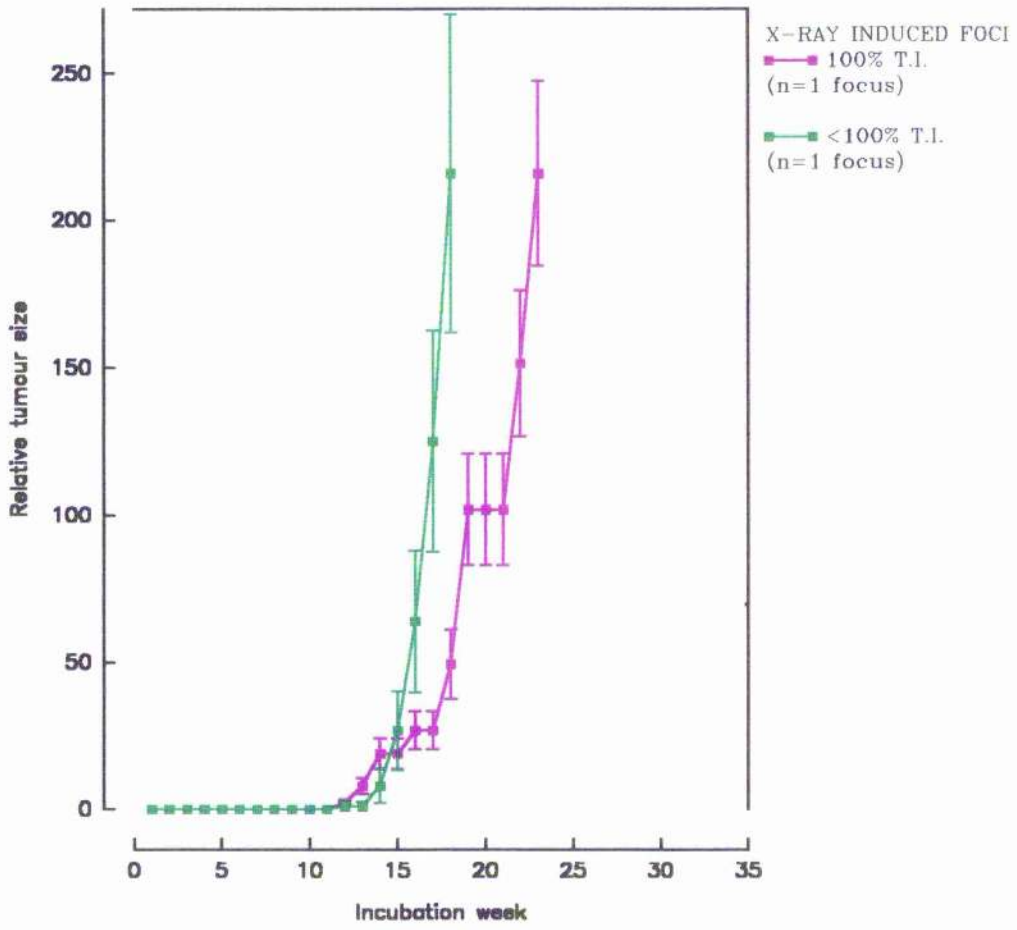
Figure 5.2.1 (d) presents data on the induction of tumours by (-) foci. The growth of the tumours produced by the alpha-particle induced (-) foci was slower for fully tumourigenic than for partially tumourigenic foci. Tumours produced by the alpha-particle induced (-) foci grew considerably slower than the equivalent X-ray induced (-) foci. Tumours were first produced by the partially tumourigenic X-ray induced (-) foci followed by the fully tumourigenic X-ray induced (-) foci, two to three weeks later. The average tumour size then grew steadily for all the X-ray induced (-) foci until a plateau was reached fourteen weeks after cell injection for the fully tumourigenic foci, these tumours started to grow again six to seven weeks later, and reached their maximum tumour size thirty-four weeks after focus cell injection. Fully tumourigenic alpha-particle induced foci produced tumours later than the X-ray equivalent, however tumours produced by the alpha-particle induced foci grew steadily and reached their maximum size before the corresponding tumours from the fully tumourigenic X-ray induced foci.

Figure 5.2.2 presents data on the growth of tumours (combined data of fully and partially tumourigenic foci) induced by the radiation-induced foci (combined data



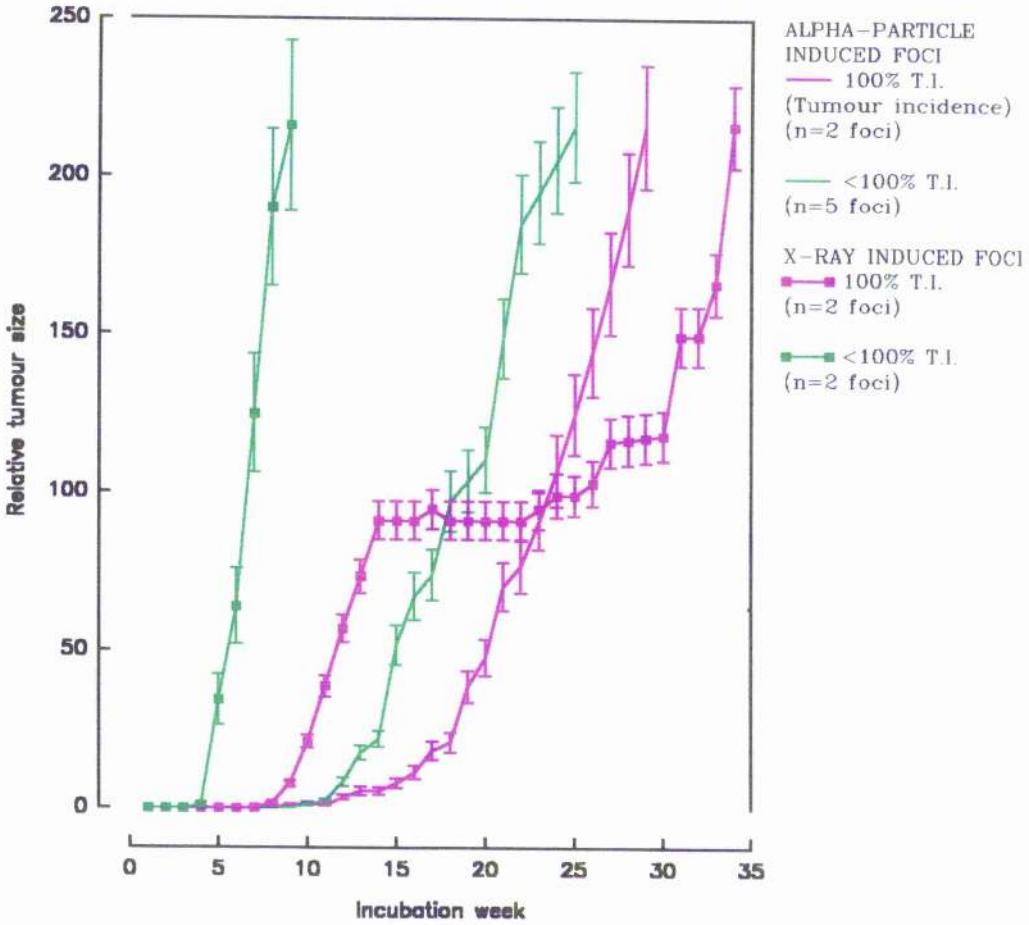
(X/+) foci

Figure 5.2.1 (b). Growth of tumours induced by radiation - induced C3H10T½ focus cells.



(X) foci

Figure 5.2.1 (c). Growth of tumours induced by radiation - induced C3H10T½ focus cells.



(-) foci

Figure 5.2.1(d). Growth of tumours induced by radiation - induced C3H10T½ focus cells.

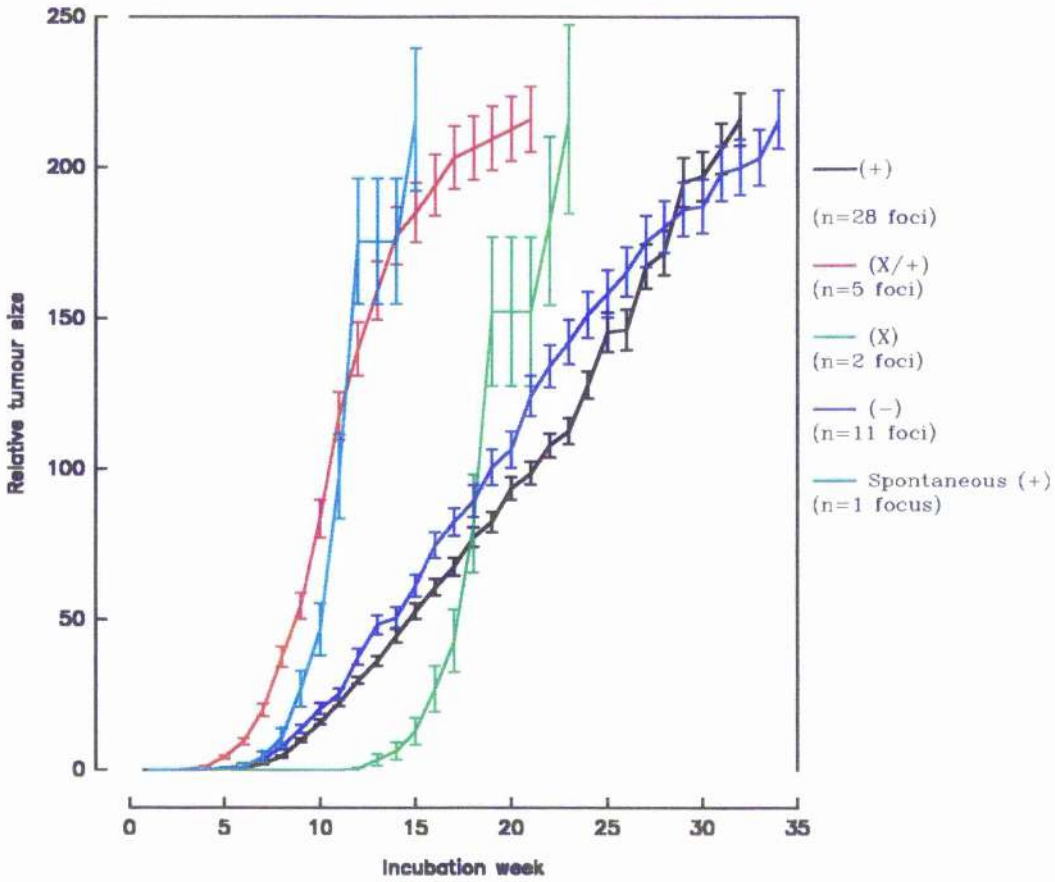
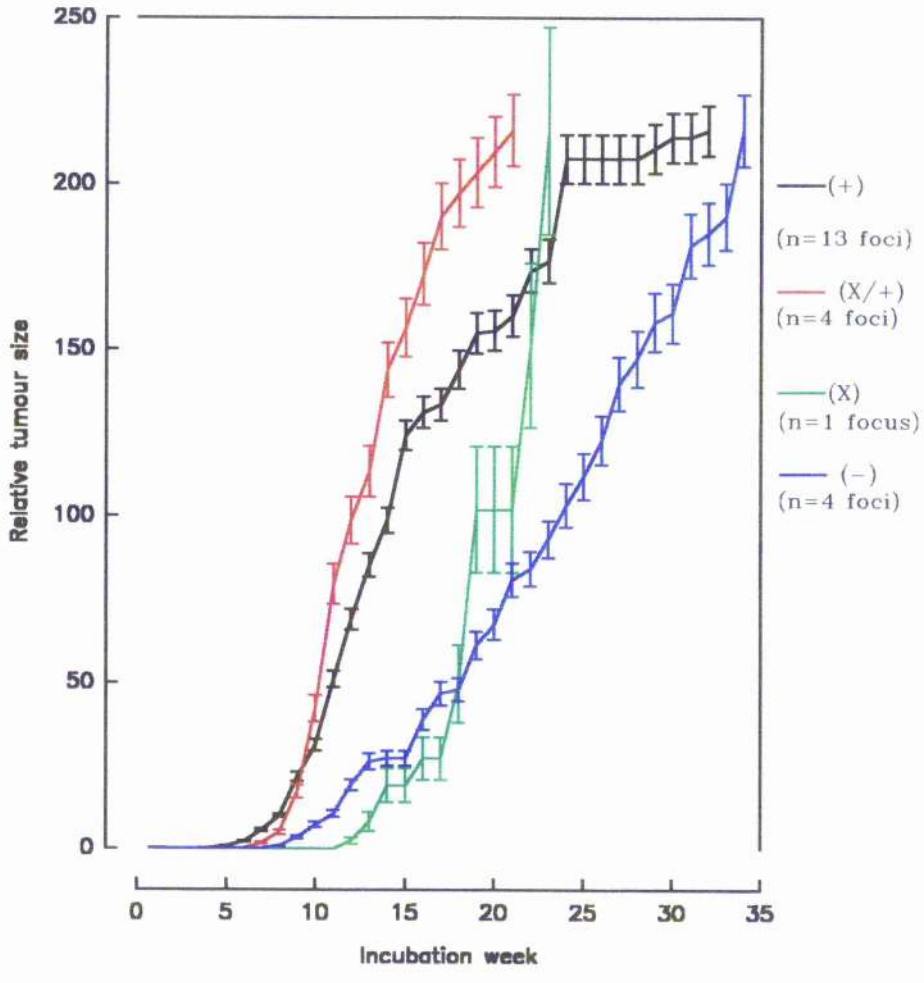


Figure 5.2.2. Growth of tumours induced by radiation - induced (combined tumourigenicity data of X-ray and alpha - particle induced foci) and spontaneously induced foci.

of the alpha-particle and X-ray induced foci). The spontaneous (+) (fully tumourigenic focus) and radiation-induced (X/+) showed comparable tumour induction and growth. Tumours from the radiation-induced foci (+) and (-) foci grew slower than the other foci with very little difference between the growth curves.

Figures 5.2.3 (a, b) present data on the growth of tumours induced by (a) fully and (b) partially tumourigenic radiation-induced foci (combined data of the alpha-particle and X-ray induced foci). As can be seen in figure 5.2.3 (a). the tumours produced by the fully tumourigenic (X/+) foci lagged by two weeks after the (+) tumours before appearing, the initial growth was rapid and the growth was comparable with that of the (+) tumours. Fully tumourigenic (-) foci produced tumours which appeared later and grew slower than any of the above tumours and did not intersect with the tumour growth curves of either the (+) or (X/+) induced tumours. In figure 5.2.3 (b) the partially tumourigenic radiation-induced foci showed very little overlap of tumour growth. (X/+) (X-ray data only available) foci produced tumours which appeared first and grew quickly, followed by the (-) foci, then the (+) foci and lastly the (X) (X-ray data only) focus. Comparison of the partially tumourigenic and fully tumourigenic (X) foci shows that both sets of tumours appeared at similar times and grew at similar rates. The same pattern was observed for the tumours induced by the (-) foci when one compares the growth of tumours induced by partially and fully tumourigenic foci.

Figures 5.2.4 (a, b) illustrate the tumourigenicity results (combined data of fully and partially tumourigenic foci) for the four focus categories induced by alpha-particles and X-rays. Of the X-ray induced foci (figure 5.2.4 (a)) the (X) foci produced tumours which were last to appear and then grew quickly, (+) foci produced tumours which appeared quickly then grew slower than any of the other categories, intersecting with the (X) curve seventeen weeks after focus cell injection and the (-) curve after twenty-five weeks. The (-) foci produced tumours which appeared in a similar time to the (+) and (X/+) tumours and grew similar to the (X/+) tumours for the most part. Of the alpha-particle induced foci (figure 5.2.4(b)), the (X/+) foci (no partially tumourigenic foci) produced the fastest growing tumours, reaching their maximum size eleven weeks before the (+) foci, while tumours from the (-) foci were the slowest to



Fully tumourigenic foci

Figure 5.2.3 (a). Growth of tumours induced by fully tumourigenic radiation - induced foci (combined data of X-ray and alpha - particle induced foci).

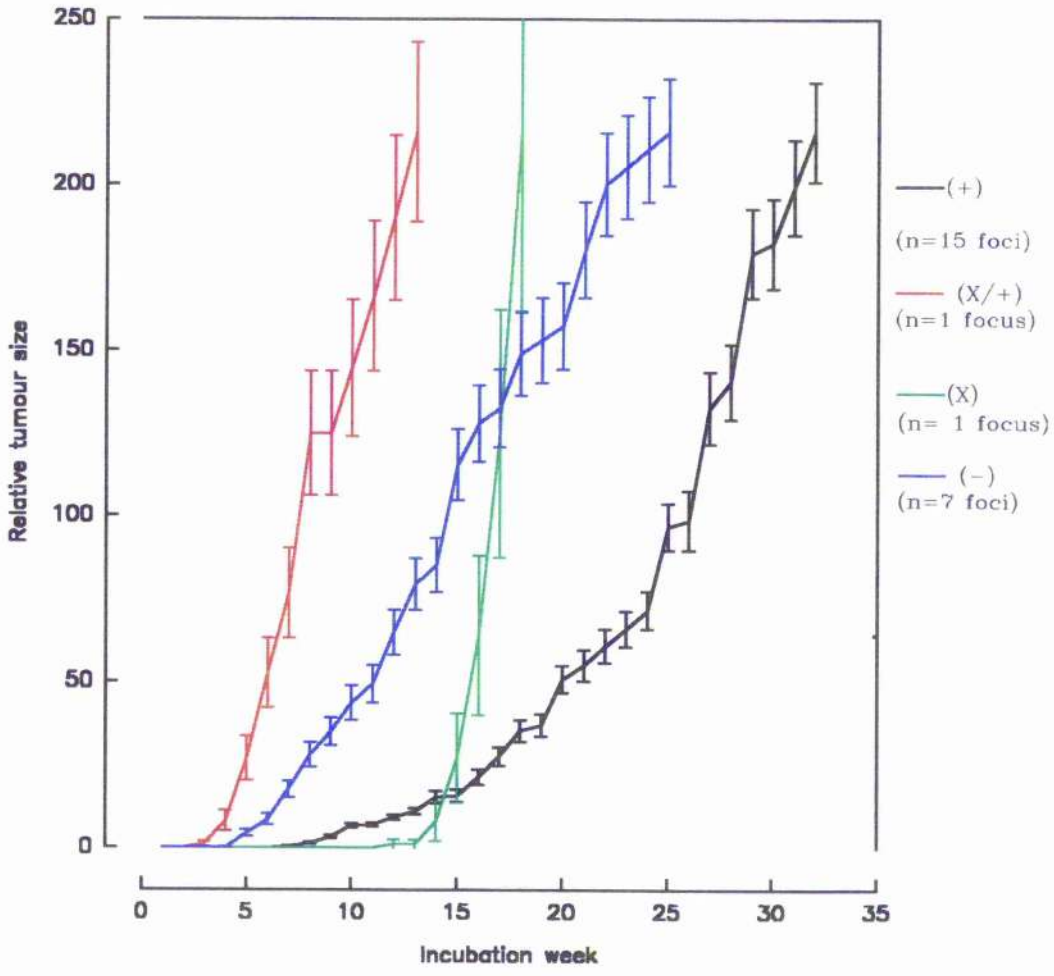
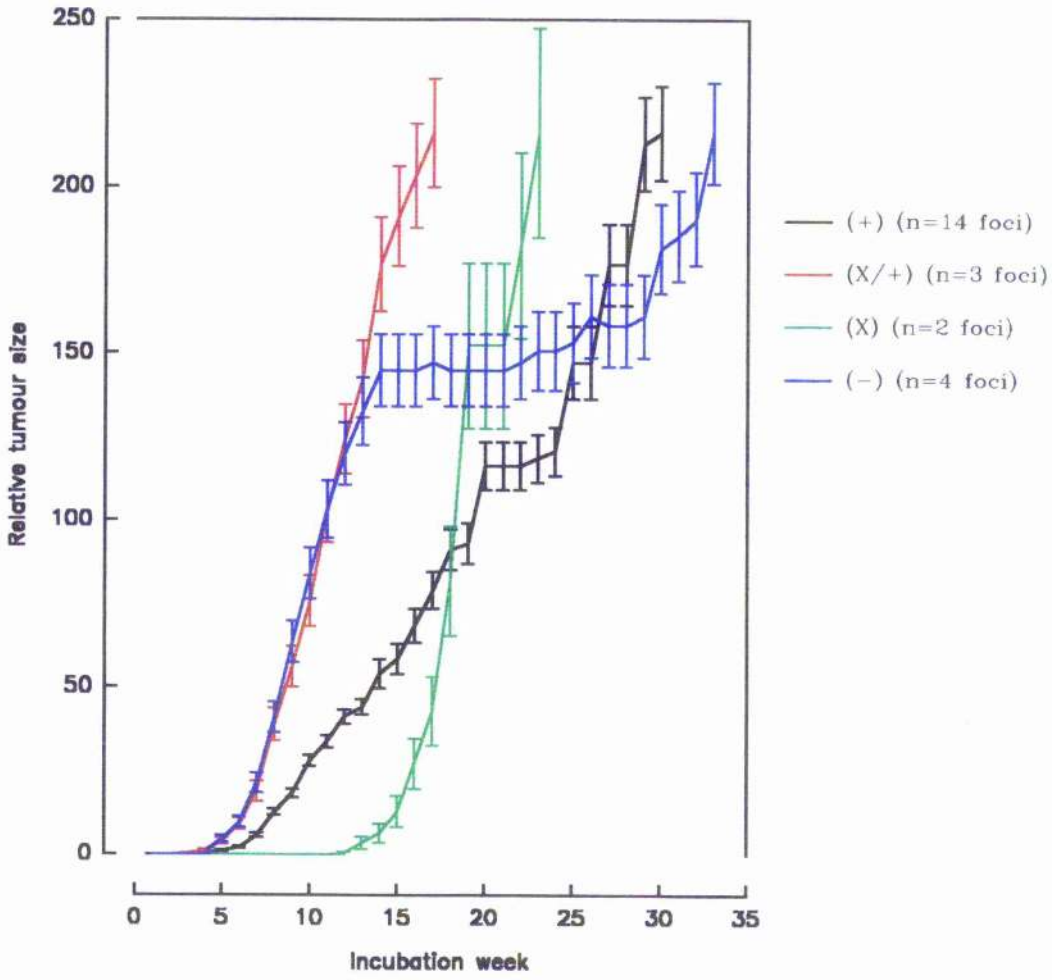
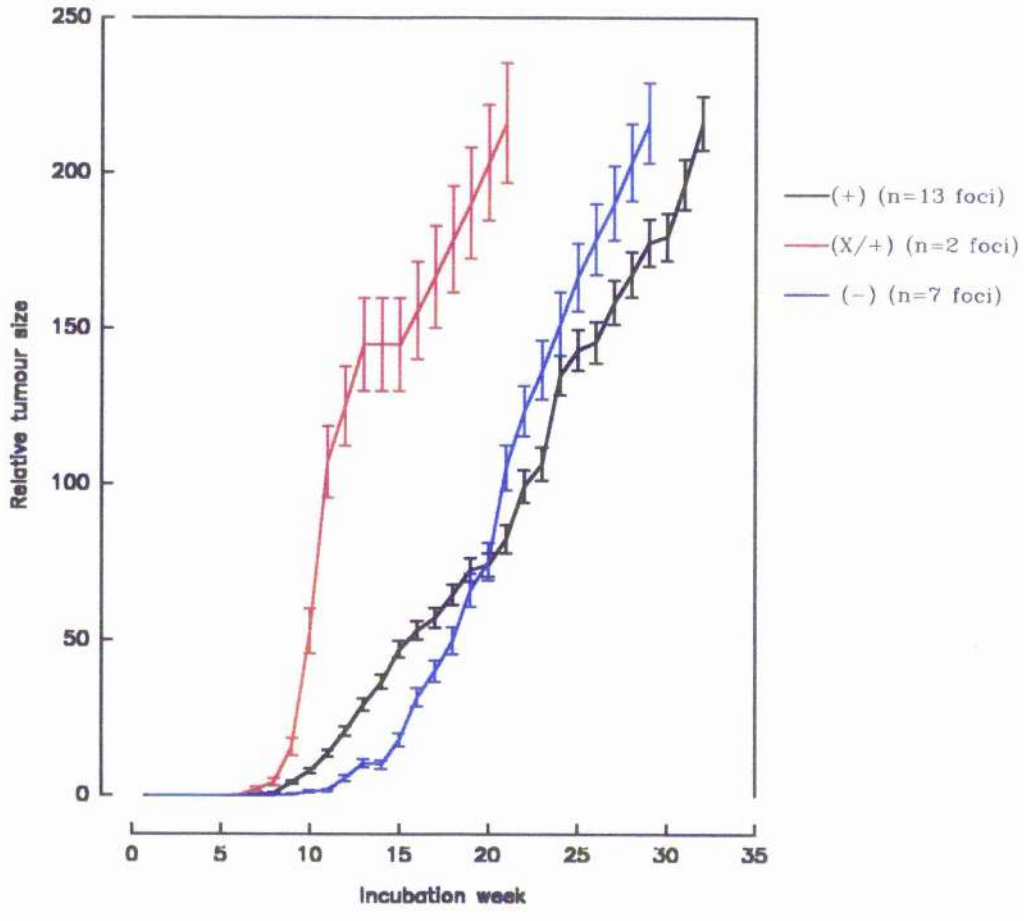


Figure 5.2.3 (b). Growth of tumours induced by partially tumourigenic radiation - induced foci (combined data of X-ray and alpha - particle induced foci).



X-ray induced foci

Figure 5.2.4 (a). Growth of tumours induced by all categories of X-ray induced C3H10T½ foci.



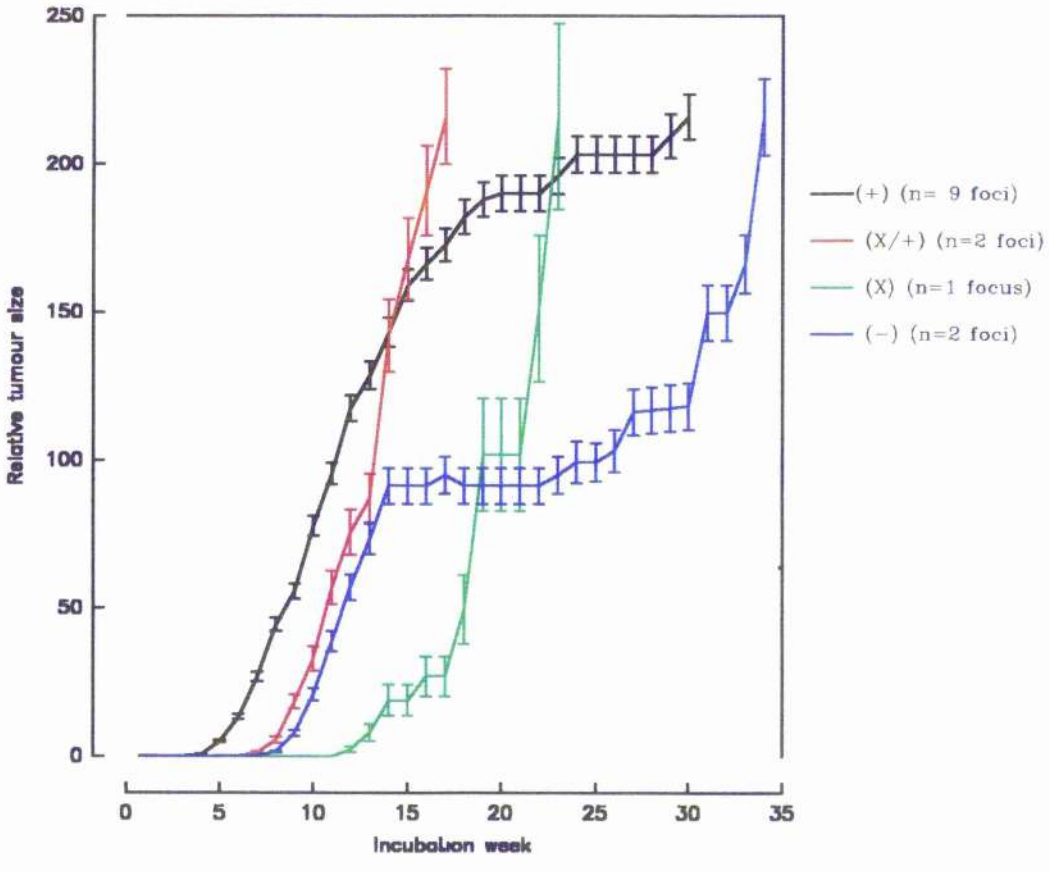
Alpha - particle induced foci

Figure 5.2.4 (b). Growth of tumours induced by all categories of alpha - particle induced C3H10T½ foci.

appear and grew following the (+) tumour growth curve, coinciding with it twenty weeks after the focus cells were injected into the mice and also reaching its maximum tumour size before the (+) foci. Comparison of the X-ray and alpha - particle data shows that the (X/+) foci induced by X-rays and alpha-particles induced tumours which grew at very similar rates. The growth curve for the tumours induced by the X-ray induced (+) foci showed an earlier induction of tumour growth than that of the alpha-particle induced foci, however the growth slowed for the tumours produced by the X-ray induced foci although the growth was still faster than for the corresponding tumours produced by the alpha-particle induced foci. The (-) foci produced tumours sooner for X-ray induced foci than for alpha - particle induced foci.

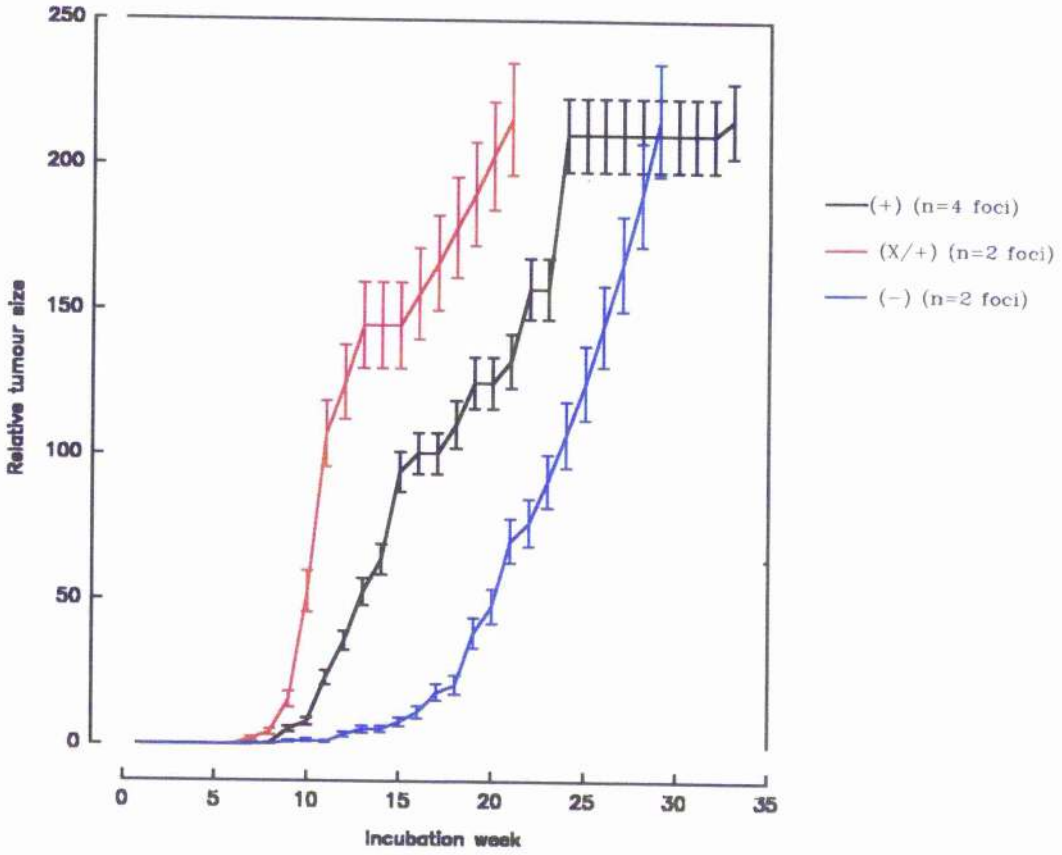
Figures 5.2.5 (a and b) present the data on the fully tumourigenic foci from the various focus categories. Tumour growth curve data for the alpha-particle induced (X/+) foci are the same data presented in figure 5.2.4 (b) since there are no data available on partially tumourigenic foci. The X-ray induced (X) focus produced tumours which were last to appear and then grew quickly, the growth reaching its maximum twenty weeks after focus cell injection, coinciding at one point with the growth curves for the tumours induced by the (+) and (-) foci. The (+) foci first produced tumours followed by the (X/+) and (-) foci. Tumours produced by the (-) and (X/+) foci appeared and grew similar to each other until about thirteen weeks after the focus cells were injected when the (-) tumour growth slowed and the (X/+) growth continued at the same rate.

Figure 5.2.6 presents the data on the partially tumourigenic foci from the various focus categories. Tumours produced by the X-ray induced (X/+) and (-) foci were the first to appear and grew quickly, reaching their maximum size by ten weeks after cell injection. The remainder of the partially tumourigenic foci produced tumours which grew significantly slower. Alpha-particle induced (+) and (-) foci and X-ray induced (+) foci produced tumours with similar growth initially, however by week twelve after the cells had been injected the growth of the (+) tumours (X-ray and alpha-particle induced) had slowed, while the alpha-particle induced (-) tumours continued to grow at the same rate, and reached the maximum tumour size twenty four weeks after focus cell injection (five weeks before the (+) tumours).



X-ray induced foci

Figure 5.2.5 (a). Growth of tumours induced by fully tumourigenic X-ray induced foci.



Alpha - particle induced foci

Figure 5.2.5 (b). Growth of tumours induced by fully tumourigenic alpha - particle induced foci.

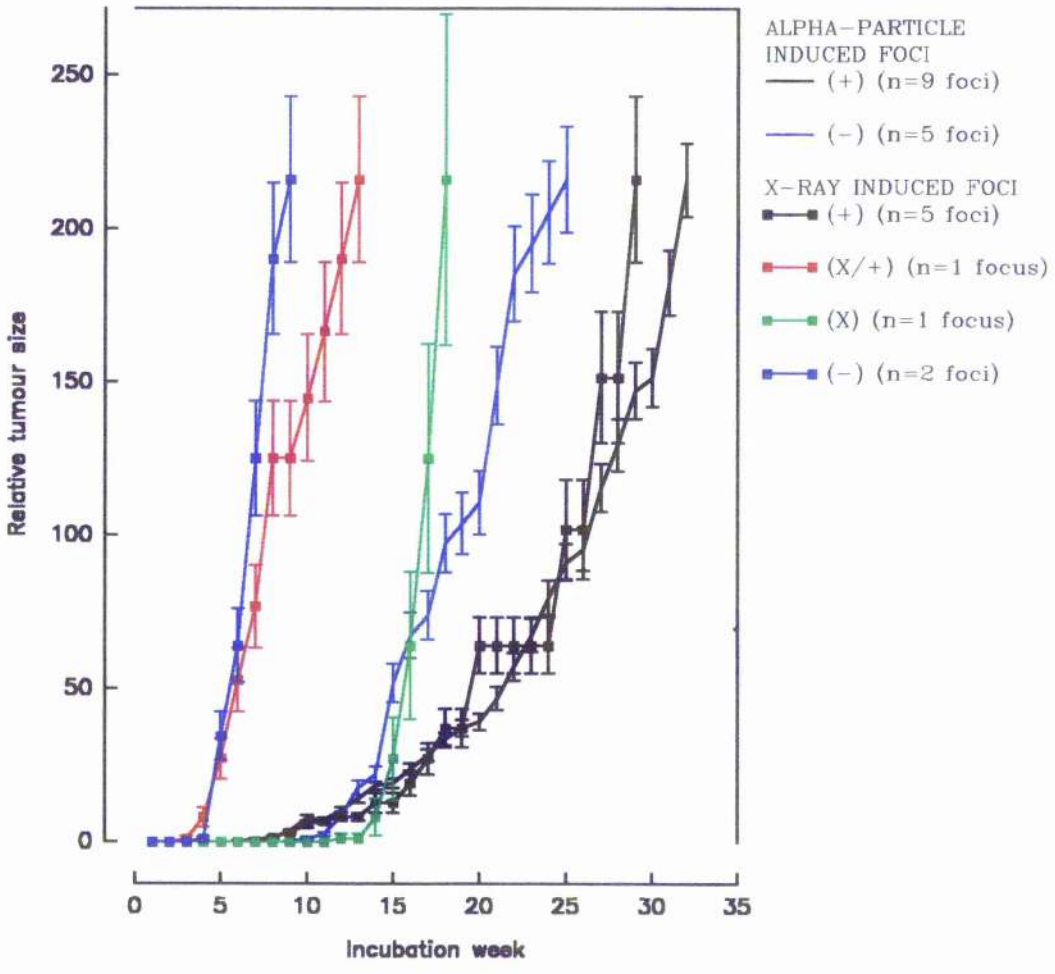


Figure 5.2.6. Growth of tumours induced by partially tumourigenic foci in all focus categories

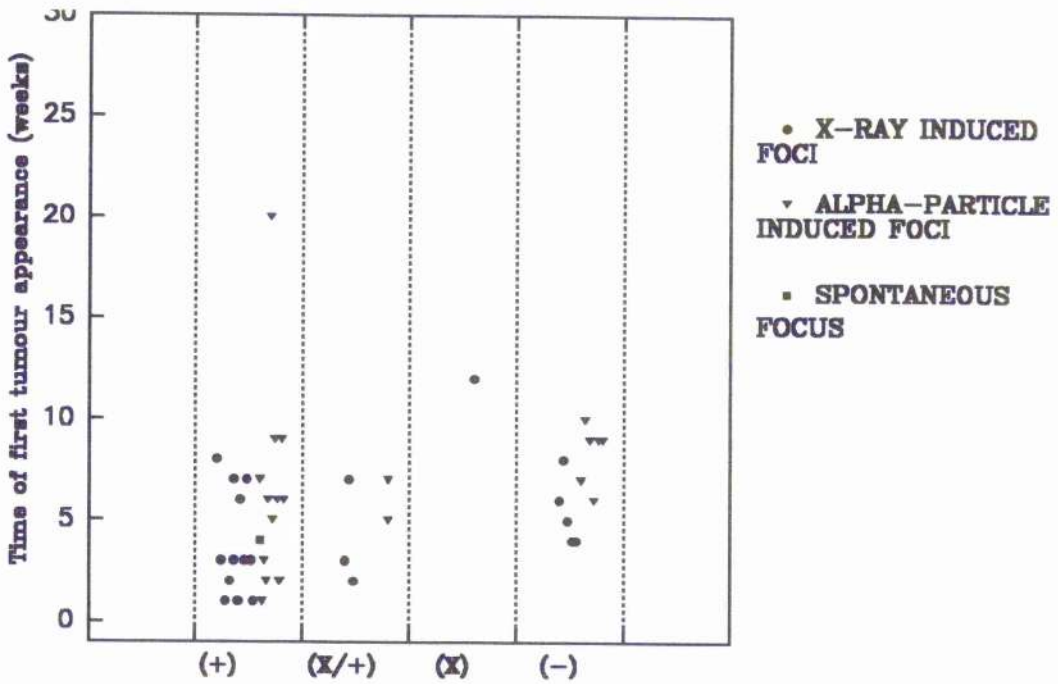
Tumour latency periods

Figures 5.2.7 (a, b) illustrate the data on the appearance of the first and last tumours induced by the various categories of foci. The majority of the (+) and all of the (-) tumours started to appear within ten weeks of the injection of the focus cells into the mice (Figure 5.2.7 (a)) Tumours induced by (X) foci (X-ray data only) started to appear twelve weeks post-injection and eight weeks post-injection for the (X/+) foci. In figure 5.2.7 (b) most of the tumours produced by the X-ray induced (+) foci had appeared by ten weeks post injection while tumours induced by the alpha-particle induced (+) foci took longer for all the tumours to appear (up to thirty - two weeks after focus cell injection). The same pattern was evident for the (-) foci with all tumours for the X-ray induced foci evident within twelve weeks of cell injection and within twenty five weeks for the alpha-particle induced foci. (X/+) foci induced all tumours within fifteen weeks of focus cell injection and the (X) foci tumours all appeared by eighteen weeks post cell injection. Foci which produced only one tumour were excluded from these figures.

Relationship of focus area and culture time of foci to tumour incidence

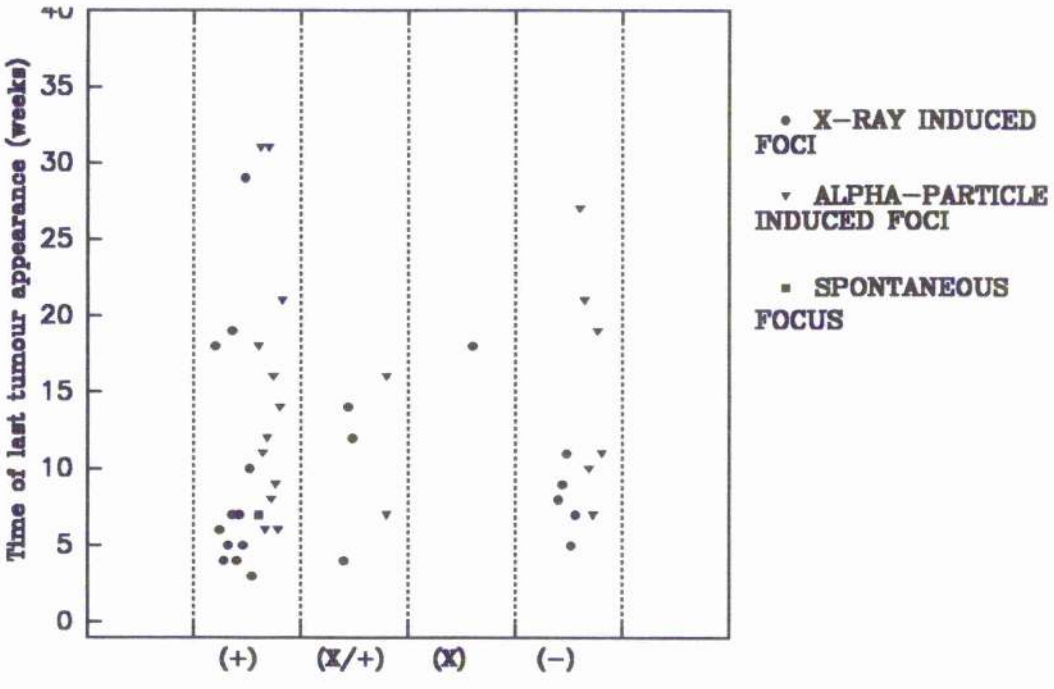
Figures 5.2.8 (a, b, c) and 5.2.9 (a, b, c) illustrate the relationship of focus area to tumour incidence of (a) radiation - induced foci (combined data of X-rays and alpha - particles), (b) X-ray and (c) alpha - particle induced foci. The foci from which the cell lines were derived varied in size and in the area of the focus isolated to produce the cell lines. Thus the initial number of cells used to develop the cell lines varied. There was no apparent dependence or correlation of the tumour incidence for the alpha-particle or X-ray data with either the total focus area (figure 5.2.8) or the area isolated (figure 5.2.9) to develop the cell line subsequently tested for tumourigenicity. The only exception to this is the possible increase of tumour incidence with focus size (total and isolated) for the X-ray induced (+) foci. It should also be noted the small number of foci tested in some categories, most notably (X/+) and (X) foci.

Figures 5.2.10 (a, b, c) illustrate the data on the tumour incidences of the various categories of foci as a function of the total time the focus cells were in culture from isolation of the cells from the foci to the time of injection of the cells into the



First tumour appearance

Figure 5.2.7 (a). Appearance of the first tumour produced by the various categories of foci induced by alpha - particles, X-rays and spontaneously.



Last tumour appearance

Figure 5.2.7 (b). Appearance of the last tumour produced by the various categories of foci induced by alpha - particles, X-rays and spontaneously.

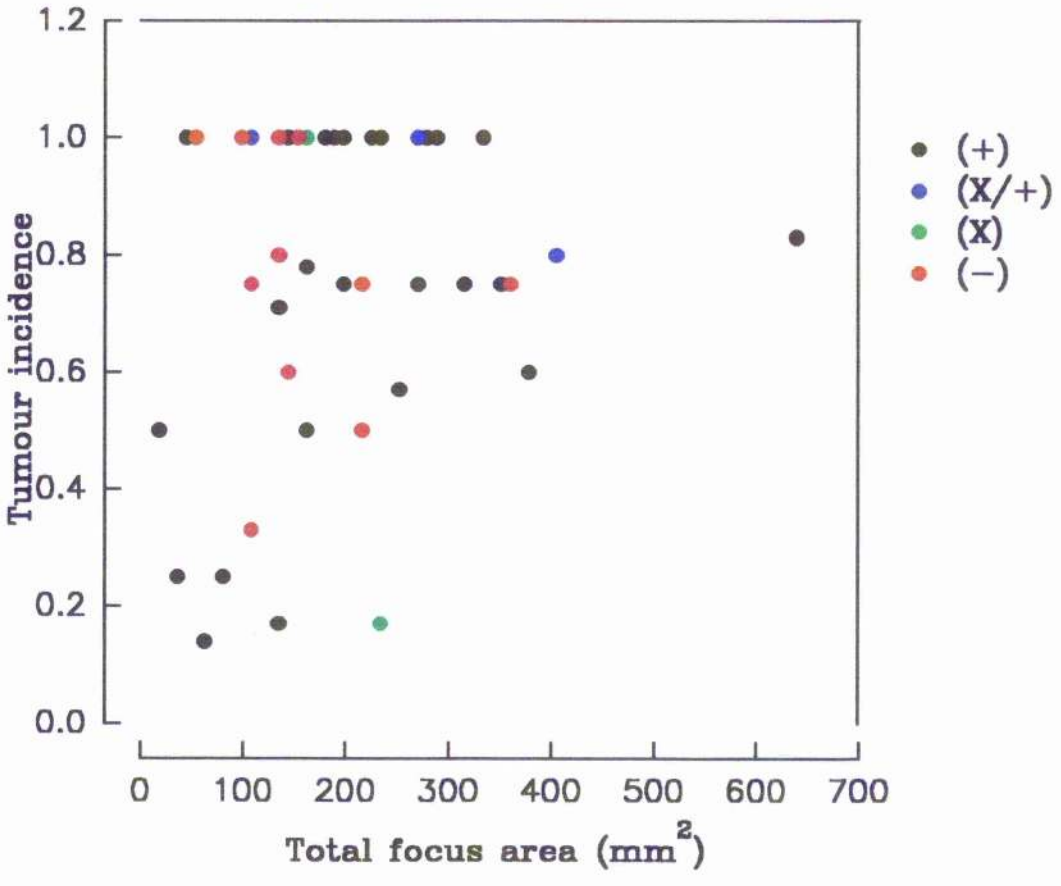


Figure 5.2.8 (a). The relationship of the tumour incidences of the various categories of foci (combined data of X-ray and alpha - particle induced foci) to the total area of the focus from which the cell lines were isolated and tested for tumourigenicity.

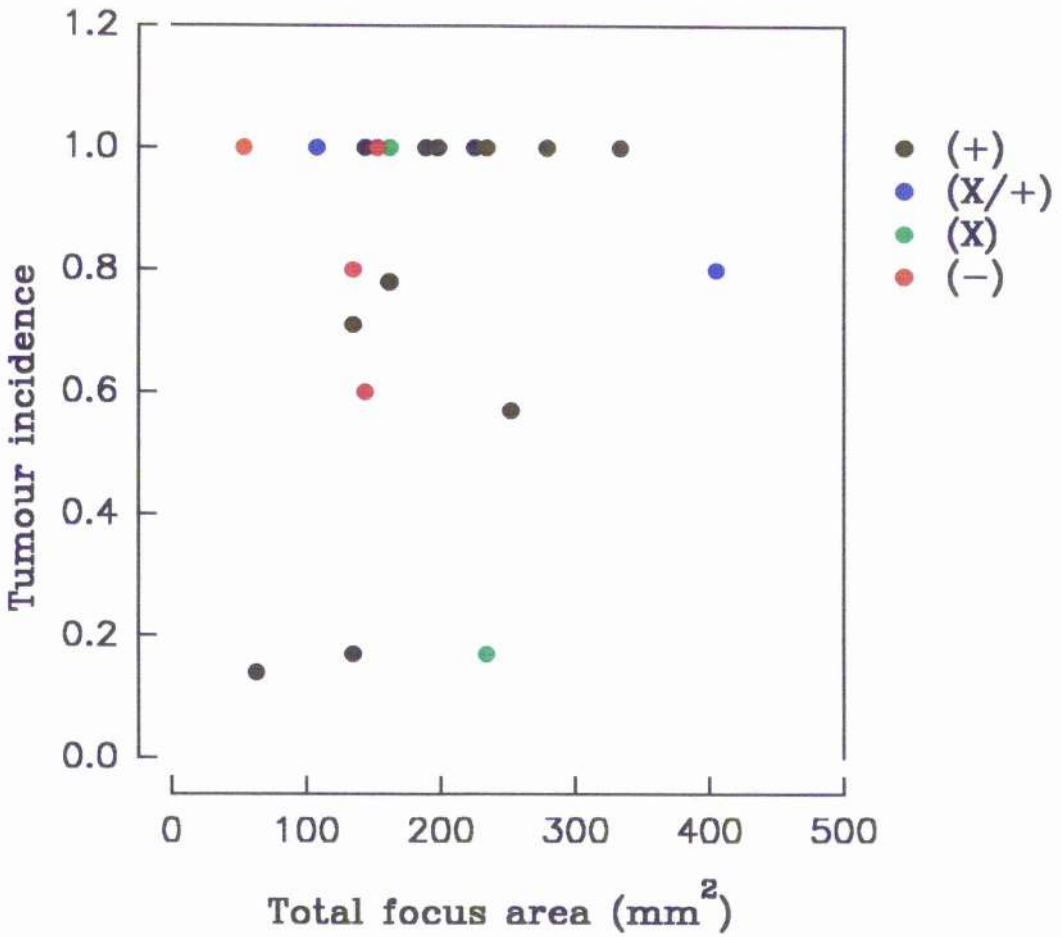
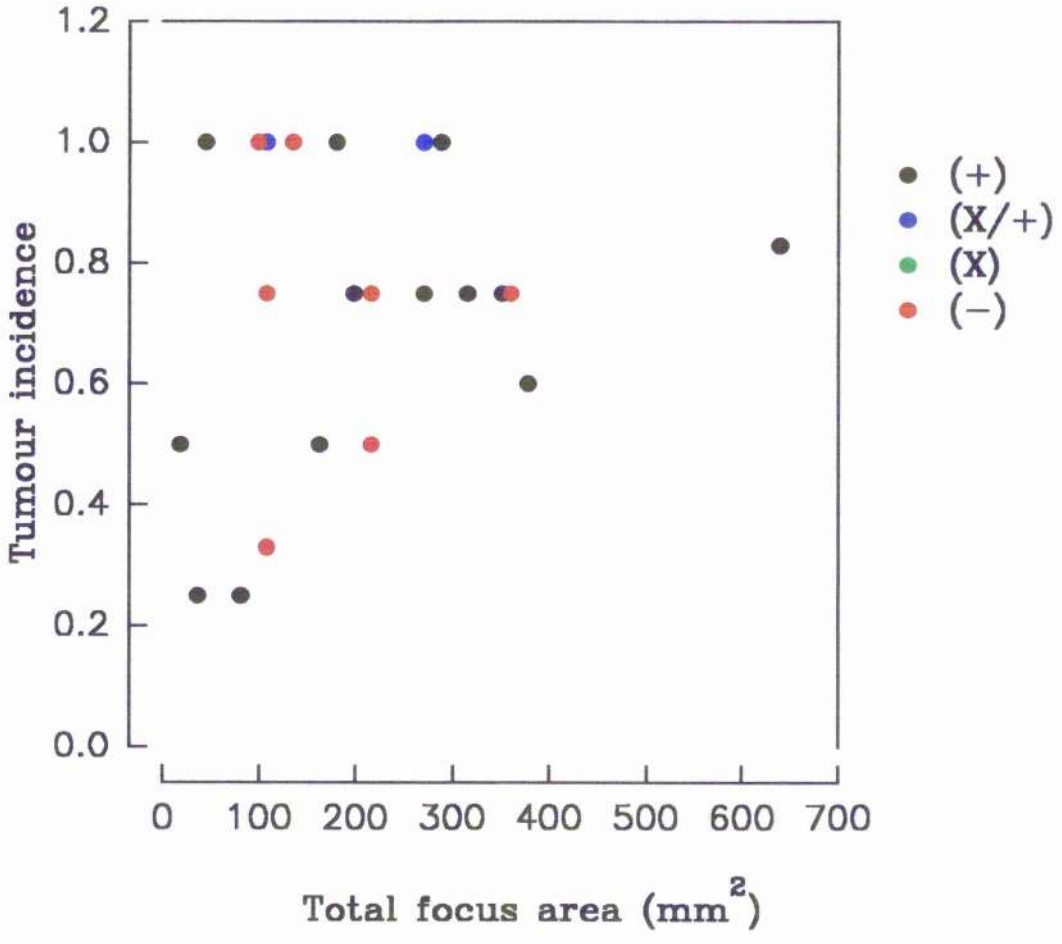
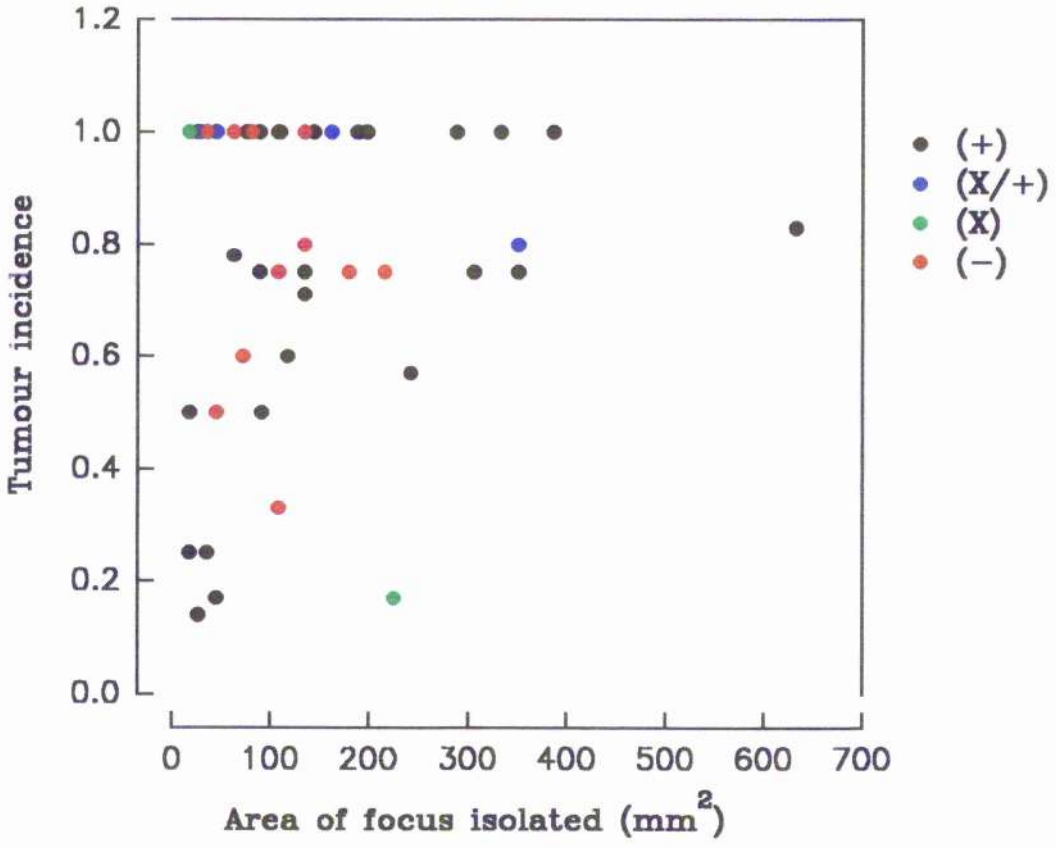


Figure 5.2.8 (b).The relationship of the tumour incidences of the various categories of X-ray induced foci to the total area of the focus from which the cell lines were isolated and tested for tumourigenicity.



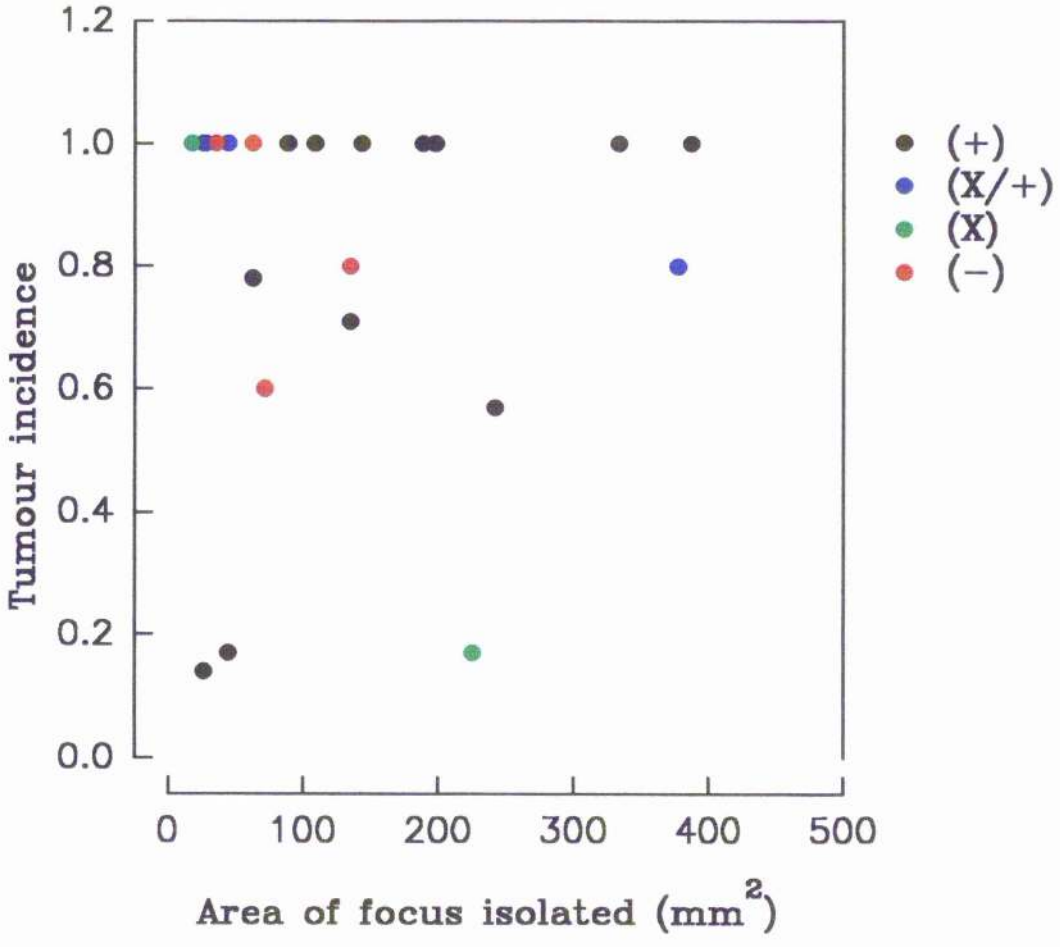
Alpha - particle induced foci

Figure 5.2.8 (c).The relationship of the tumour incidences of the various categories of alpha - particle induced foci to the total area of the focus from which the cell lines were isolated and tested for tumourigenicity.



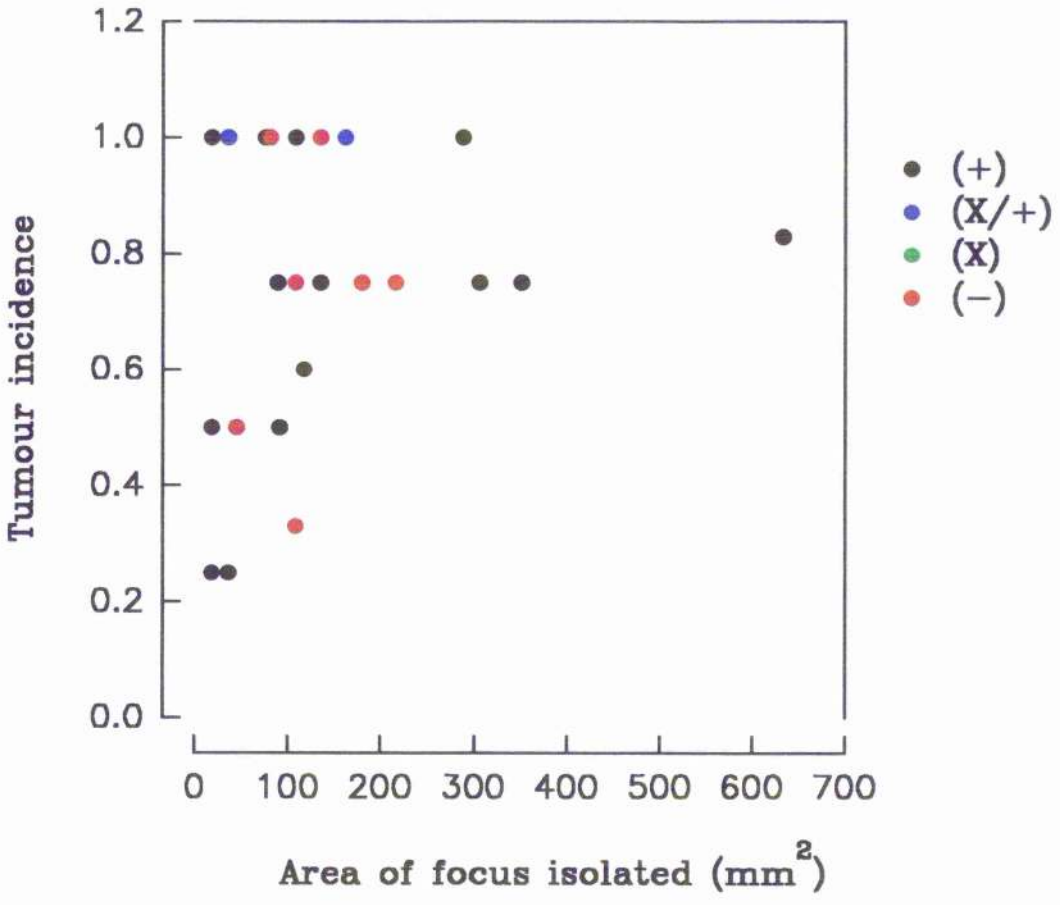
Combined radiation data

Figure 5.2.9 (a). The relationship of the tumour incidences of the various categories of foci (combined data of X-ray and alpha - particle induced foci) to the area of focus isolated to establish the cell lines tested for tumourigenicity.



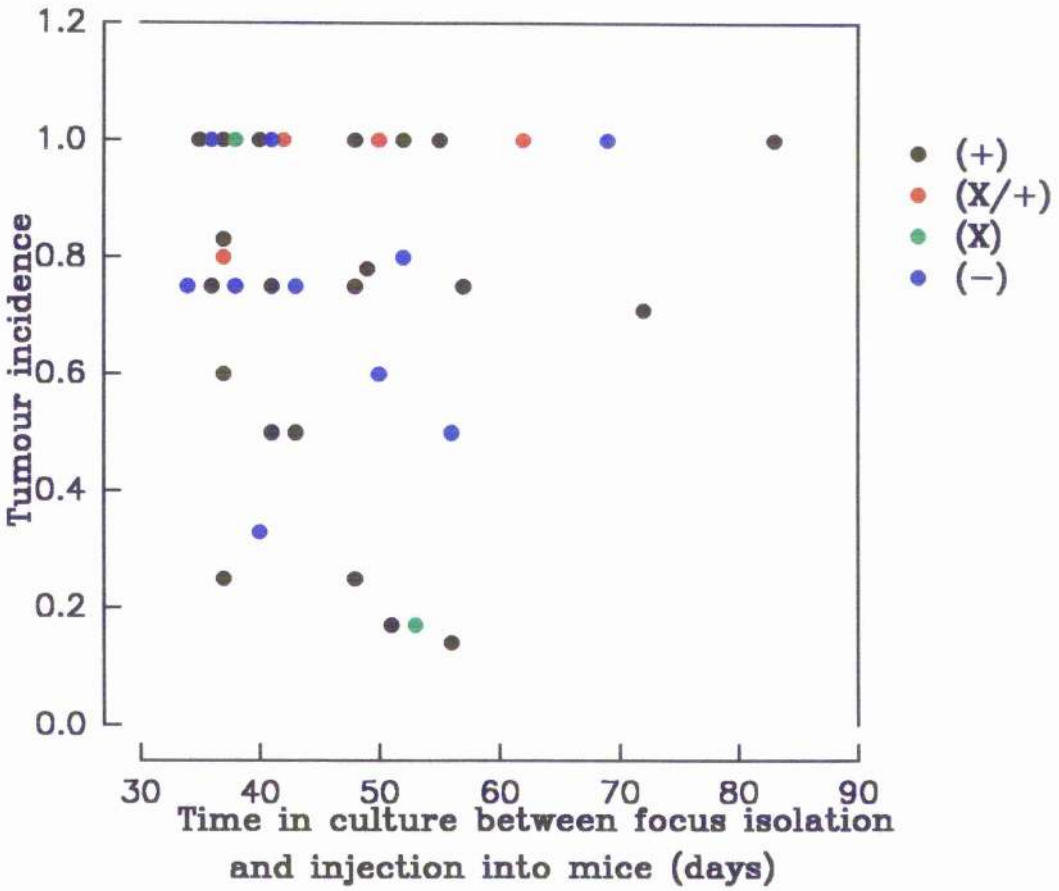
X-ray induced foci

Figure 5.2.9 (b).The relationship of the tumour incidences of the various categories of X-ray induced foci to the area of focus isolated to establish the cell lines tested for tumourigenicity.



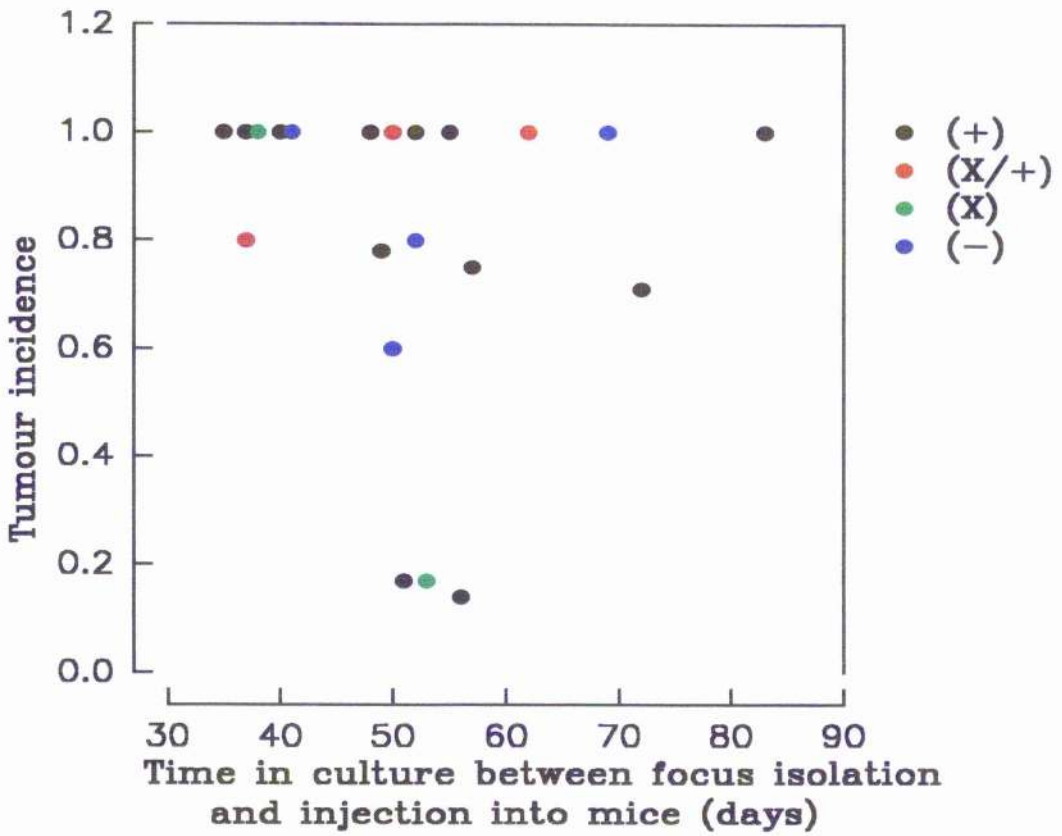
Alpha - particle induced foci

Figure 5.2.9 (c).The relationship of the tumour incidences of the various categories of alpha - particle induced foci to the area of focus isolated to establish the cell lines tested for tumourigenicity.



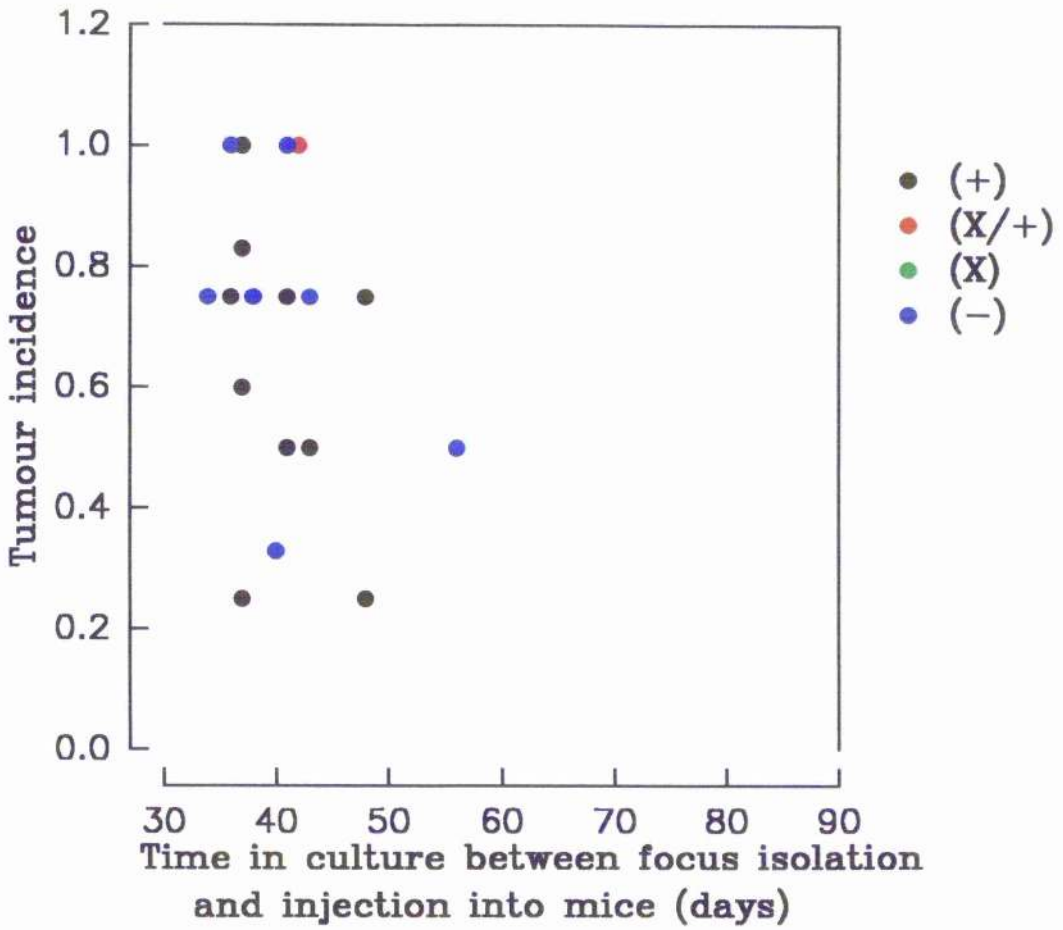
Combined radiation data

Figure 5.2.10 (a). The relationship of the tumour incidence of the various categories of foci (combined data of X-ray and alpha - particle induced foci) to the total number of days the cells were in culture between focus isolation and injection for tumourigenicity testing.



X-ray induced foci

Figure 5.2.10 (b). The relationship of the tumour incidence of the various categories of X-ray induced foci to the total number of days the cells were in culture between focus isolation and injection for tumourigenicity testing.



Alpha - particle induced foci

Figure 5.2.10 (c). The relationship of the tumour incidence of the various categories of alpha - particle induced foci to the total number of days the cells were in culture between focus isolation and injection for tumourigenicity testing.

C3H mice for tumourigenicity testing. The total culture time comprised of two periods in culture , firstly from isolation of the cells from the focus to storage of the cells in liquid nitrogen (described in section 2.5.6) and secondly from retrieval of the cells from storage to subcutaneous injection in the mice. No correlation was evident between the tumour incidences and the time the cells were in culture.

Discussion

Tumour incidence

It is evident from table 5.2.2. that a greater proportion of the X-ray induced foci were tumourigenic in all focus categories while the majority of the alpha-particle induced foci in each category were not tumourigenic. This was true whether the foci were classified by the author alone or by the European collaborative group. The data indicate a stricter classification by the author of (+) transformed foci, compared to the European collaborative group, many of the foci classified as (X) foci by the author were categorised as (X/+) by the collaborative group. This resulted in a lower fraction of tumourigenic (+) foci according to the author's classifications for the X-ray induced foci but the same fraction of tumourigenic alpha-particle induced (+) foci, compared to the European collaborative group's classifications. These differences in classifications and the impact on the tumourigenic fractions of the various focus categories highlight the subjective nature of the C3H10T½ transformation assay and a feature common to many biological systems. Since a group of at least eight people examined and categorised the foci as part of the collaborative effort, it was the collaborative classifications which were used for the graphical presentation of the data.

Data presented in tables 5.2.4. and 5.2.5. and in figures 5.2.8. and 5.2.9. show that most of the tumourigenic foci produced the first tumour within ten weeks of injection of the focus cells into the C3H mice. The exceptions were the X-ray induced (X) foci (no alpha-particle induced (X) foci) which first showed tumours at twelve weeks after injection of the focus cells. The time after injection for all tumours to be evident varied between focus categories. Many of the X-ray induced (+) foci had produced all tumours within twenty weeks of injection of focus cells into the mice, while most of the alpha-particle induced foci required more time (up to thirty-two weeks). All tumours from the X-ray induced (-) foci were evident within twelve weeks of injection of the focus cells, while those from the corresponding alpha-particle induced foci took twice as long. The foci in the remaining categories ((X/+): X-ray and alpha-particle induced and (X) (X-ray data only)) required fifteen to eighteen weeks to produce all tumours.

The foci from which the cells tested for tumourigenicity were isolated varied in size, both in the total area of the focus and in the area isolated for development of the cell line. The influence of the consequent variation in the initial number of focus cells was unknown but there appears to be no direct correlation between the tumour incidence and the focus area (total or isolated).

Table 5.2.1. presents the variation in the tumourigenicity data obtained by other authors using a similar process of isolating cells from C3H10T½ foci transformed by different transforming agents and expanding the cell population to sufficient numbers to test for tumourigenicity. The tumourigenicity of type II foci varied from 14% - 100% and type III from 0% - 100% and the first publication on C3H10T½ foci (Reznikoff *et al.* 1973) reported 50% tumour incidence for type II foci and 80% for type III. This variation in the tumour incidence was also seen in the present data (table 5.2.3.) when the foci were placed in the categories of type I, II, and III as described by Reznikoff *et al.* (1973) and including the type X category. The foci were classified into types I, II, III, and X in two ways. The first method was the routine method of focus classification in laboratories using the C3H10T½ transformation assay where the foci are categorised according to a 'scale of importance' for determination of positively or negatively transformed foci. All foci contained a mixture of characteristics of the various categories, type I, II, III, or X. While some foci were clearly of one specific type, many were borderline foci and emphasis was placed on one type or other for classification. For these studies the borderline foci were classified according to a scale of decreasing importance from type III to II to X to I (routine method of classification of transformed foci). However it was also decided to use a second method of classification and categorise the foci into both categories it contained characteristics of, thus a focus of mixed type X and II characteristics was classified in both categories, type X and II, but the critical classification was type II according to the above 'scale of importance'.

As previously stated, the tumour incidence of the X-ray induced foci was higher than that of the alpha-particle induced foci in all categories, for both methods of focus classification. The tumourigenicity of X-ray induced foci was higher for type II foci and lower for type III foci than reported by Reznikoff *et al.* in 1973 for

chemically induced transformants, while the alpha-particle induced foci tumourigenicity was lower for both categories. Tumourigenicity of type X foci was between that reported for type II and III foci for radiation-induced foci, examined together or separately as X-ray versus alpha-particles. When the data from the X-ray and alpha-particle induced foci were combined the type II tumourigenicity was still higher than reported by Reznikoff *et al.* while the type III tumourigenicity was considerably lower and the type X data were closer to that reported for type III tumourigenicity.

Type I foci were reported as non-tumourigenic by Reznikoff *et al.* (1973) and few reports have been found where other authors have isolated type I foci to test the tumourigenicity. Aside from the possibility of misclassification the relatively high tumour incidence of the type I foci (table 5.2.3.) may be explained if one assumes that type I foci are precursors of type II and type III and that some partially transformed cells became transformed in the time taken for the cells to be isolated from the focus, subcultured and the cell number increased for subsequent injection into the C3H mice. However preliminary data on work in progress indicate that this may not be the case, as non-tumourigenic type I foci and unirradiated parent C3H10T½ cells were expanded several generations in culture and tested for tumourigenicity at twenty, thirty, and forty passages (up to approximately twenty weeks in culture) after focus cell isolation. To date no tumours have been found. This does not preclude type I foci being precursors of type II and III foci, it suggests that if this is the case then additional steps are required other than extended passage in culture for the type I to proceed to type II and / or III. These 'steps' could be changes in the levels of oncogenes or tumour suppressors genes or alternatively an epigenetic alteration, or any combination of these, the identity of the possible changes involved in C3H10T½ transformed cells compared to normal cells is not clear (for example, Borek *et al.* 1987, Privitera *et al.* 1990, Krolewski *et al.* 1994). It is worth noting that only two type I foci were identified and cell lines developed (foci labelled as X14 and X18 in table 5.2.4.). These foci were very similar in appearance and both foci were isolated from culture dishes containing other foci (most classified as type I foci, a type X was also observed) of very similar characteristics. It was speculated that focus cells detached

and relocated from one central focus to areas of the culture dish, where other foci then developed, and these events correlated with a similar pattern observed *in vivo* of tumour cell metastasis, and that this ability of focus cells possibly indicated a malignant nature. Thus the tumourigenicity data of foci isolated from cultures containing more than one focus were re-examined. Data displayed a greater proportion of tumourigenic foci when foci on multifocus culture flasks were considered, than when data on all foci were included: eighty-five percent of X-ray induced foci (eleven of thirteen foci) compared to seventy-eight percent (twenty-one of twenty-seven foci) when all foci were included, fifty percent of alpha-particle induced foci (eighteen of thirty-eight foci) compared to thirty-eight percent (twenty-three of sixty foci) when data on all foci were included.

No publications have been found reporting data on a focus similar to that designated as type X in the work presented here. The data demonstrate that the majority of the foci with X focus characteristics were tumourigenic. The X focus characteristics were most often found in the presence of some criss-crossing cells and thus were classified as (X/+) in this work and most probably as type III foci by other authors.

Tumour growth

Comparison of the growth of the tumours induced by the various categories of foci ((+), (X/+), (X), (-)) revealed that for the radiation-induced foci (combined data of X-ray and alpha-particle induced foci, fully and partially tumourigenic foci) the (X/+) foci produced the fastest growing tumours while the (+) and (-) foci produced tumours showing a more gradual increase in size. When these data were then examined as fully tumourigenic versus partially tumourigenic foci, the (X/+) foci showed the same pattern of tumour induction and growth described above. Differences between the other focus categories was enhanced by the contribution of the partially tumourigenic foci, where the (+) foci produced tumours with slower growth and the tumours induced by the (X/+) and (-) foci grew in a similar pattern but (X/+) tumours appeared first. Fully tumourigenic (-) foci produced tumours with a slower growth than the tumours induced by either the (+) or (X/+) foci which displayed comparable

growth. Comparison of the (X) foci was not possible as there were no tumourigenic alpha-particle induced (X) foci.

Further breakdown of the data on the radiation-induced foci into alpha-particle versus X-ray induced foci (combined data of fully and partially tumourigenic foci) revealed that the (X/+) foci induced tumours with similar growth curves irrespective of the radiation type, which were also comparable to the tumours induced by the spontaneous (+) focus. The X-ray induced (+) and (-) foci produced tumours which appeared earlier than those induced by the corresponding alpha-particle induced focus categories, but the corresponding growth curves of alpha-particle versus X-ray coincided at a later time in the incubation period. Further analysis of these data by examination of the fully tumourigenic versus partially tumourigenic foci displayed yet again that the (X/+) foci induced tumours with similar growth curves irrespective of whether the focus was fully or partially tumourigenic. Data were not available for a partially tumourigenic alpha-particle induced (X/+) focus. The fully tumourigenic X-ray induced (+) and (-) foci followed the pattern described above and produced tumours which appeared earlier than those induced by the corresponding alpha-particle induced focus categories. There was no significant difference between the growth curves of the tumours induced by the partially tumourigenic (+) foci (X-ray versus alpha-particle). The partially tumourigenic X-ray induced (-) foci produced tumours five weeks before the alpha-particle induced (-) foci and the growth curves remained separate.

Examination of the individual focus categories presents that for the (+) foci (X-ray or alpha-particle induced) the partially tumourigenic foci generally produced tumours which grew slower than those produced by fully tumourigenic foci. Fully tumourigenic X-ray induced (+) foci produced tumours which appeared earlier than those of the corresponding alpha-particle induced foci, however the growth curves later crossed. This pattern was also seen in the total tumourigenicity data. No great differences were observed between the radiation types for the tumour growth curves of the partially tumourigenic (+) foci. Partially tumourigenic (-) foci produced tumours which grew faster than those produced by the fully tumourigenic foci for both radiation types. Tumour growth for the alpha-particle induced (-) foci was slower than

for the corresponding X-ray induced foci. Data for the (X) foci were only available for the X-ray induced foci and there was no significance difference between the tumour growth curves of the partially tumourigenic foci and the fully tumourigenic foci. X-ray induced partially tumourigenic (X/+) foci produced tumours which appeared before tumours from the fully tumourigenic foci (no partially tumourigenic alpha-particle induced (X/+)). There was no notable difference between the radiation types for the fully tumourigenic (X/+) foci.

DNA damage and genomic instability

There is a wide range of evidence that many of the effects of radiation are due to its ability to damage DNA, for example by inducing double strand breaks (studies summarised in UNSCEAR 1993). High-LET radiation damage is generally considered to be qualitatively different to that of low-LET radiation damage. One possibility for these differences is the pattern of energy deposition of the various radiation types. High-LET radiation such as alpha-particles deposit their energy in a much more localised area within the cell nucleus than low-LET radiation such as X-rays. Even if the same amount of damage is induced, the cells' repair mechanism has more time to correct the damage if it is spaced apart at a sufficient distance to prevent interaction of two or more damaged sites (Brenner 1990). The clustering of damage induced by high-LET radiation increases the probability of molecular and cellular consequences (Goodhead 1989, Ward 1994). Several studies report that high-LET radiation damage is not as efficiently repaired as that produced by low-LET radiation (Robertson *et al.* 1983, Loucas and Geard 1994). The misrepair of DNA damage such as strand breaks may have several consequences including lethality or transformation for the affected cell. There is increasing evidence that high LET radiation induces an instability in the genome which may take several generations to manifest itself. Two studies in particular report genome instability induced by alpha-particles but not by X-rays (Aghamohammadi *et al.* 1988, Kadhim *et al.* 1995). Marder and Morgan's data (1993) using a hamster human hybrid cell line disputes these data by illustrating chromosomal instability induced by X-rays. Aghamohammadi *et al.* in their studies of the induction of sister chromatid exchanges in lymphocytes favoured the explanation for their results

that the alpha-particle induced DNA lesions were retained longer than those induced by X-rays and the lesions were then carried into the S-phase of the cell cycle where they could be converted into sister chromatid exchanges. The kinetics of induction of sister chromatid exchanges by alpha-particles and X-rays bear certain similarities to the induction of transformation (Nagasawa *et al.* 1990, Marder and Morgan 1993).

Data presented in this thesis on the tumourigenicity of X-ray and alpha-particle induced transformed foci isolated from the C3H10T½ transformation assay could be explained by the induction of chromosomal instability by high-LET radiation and to a lesser extent by the low-LET radiation. Transformation may be regarded as a step on the route to carcinogenesis of a normal cell. Although the radiations induced sufficient damage to induce transformation in the parent cells from which the cell lines tested for tumourigenicity were developed, this damage may not have been sufficient in all cases to produce tumourigenic transformants. Alpha-particle induced foci were less tumourigenic than the X-ray induced foci, under the conditions of this assay, and where tumours developed they appeared later than those produced by the X-ray induced foci. The alpha-particles may have induced instability in the focus parent cells genome at the time of irradiation which takes a considerable period of time to manifest itself. This instability may manifest itself as aberrations before or after focus formation leading to tumourigenicity or the aberrations may prove lethal to the focus cells, making them non - viable and thus resulting in an increase in cell death and a lower tumour incidence. It is also possible that given a longer period of incubation of the cells in the mice the alpha-particle induced foci would reach the same level of tumourigenicity as the X-ray induced foci.

Summary

The data presented in this section illustrate that a higher proportion of the X-ray induced foci were tumourigenic while most of the alpha-particle induced foci were non - tumourigenic. This applied to (+), (X/+), (X) and (-) foci and was the case irrespective of whether the foci were classified solely by the author or by the European Collaboration group. Generally the X-ray induced foci produced tumours earlier and required less time for all tumours to appear than the alpha-particle induced foci. There appears to be no correlation between the tumour incidence and the focus area (total or only the area isolated) or the time the focus cells spent in culture prior to tumourigenicity testing. Comparison of the growth of the tumours indicated that the (X/+) foci generally produced the fastest growing tumours while the (+) and (-) foci showed slower growth of tumours. However a much smaller number of foci were examined in the (X/+) category than in the (+) or (-) focus categories. Examination of the partially and fully tumourigenic foci revealed that in general tumours from the partially tumourigenic foci grew slower or appeared later than those of the fully tumourigenic foci. The above data are discussed again in conjunction with the data on the focus reconstruction studies, growth parameters and cytogenetics in the final discussion at the end of this chapter.

Appendices

Relative tumour sizes (mean ± standard error) of tumours induced by fully tumourigenic, partially tumourigenic and combined total of the α-particle induced foci							
	(+)			(X/+)	(-)		
Incubation week	Total	Full	Partial	Full	Total	Full	Partial
1	<0.1 ± 0.1	<0.1 ± 0.1	0	0	0	0	0
2	<0.1 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1	0	<0.1 ± 0.1	0	<0.1 ± 0.1
3	<0.1 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1	0	<0.1 ± 0.1	0	<0.1 ± 0.1
4	<0.1 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1	0	0	0	0
5	<0.1 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1	0	0	0
6	<0.1 ± 0.1	<0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.07	<0.1 ± 0.1	0	<0.1 ± 0.1
7	0.4 ± 0.1	0.2 ± 0.1	0.8 ± 0.1	2 ± 0.7	<0.1 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1
8	0.8 ± 0.1	0.4 ± 0.1	1.3 ± 0.2	4.3 ± 1.1	0.1 ± 0.1	0.3 ± 0.2	<0.1 ± 0.1
9	4.3 ± 0.5	5.4 ± 1	3.4 ± 0.4	15.6 ± 2.8	0.5 ± 0.2	1 ± 0.4	0.1 ± 0.1
10	7.9 ± 0.7	8 ± 1.2	7.8 ± 0.8	52.7 ± 7.2	1.2 ± 0.3	1.3 ± 0.5	1.1 ± 0.4
11	13.5 ± 1.0	23.1 ± 2.4	7 ± 0.8	107.2 ± 11.5	1.6 ± 0.4	1 ± 0.3	2.4 ± 0.6
12	20.6 ± 1.4	35.9 ± 3.3	10.2 ± 1	125 ± 12.8	1.5 ± 0.8	3.4 ± 0.9	8.4 ± 1.4
13	29.2 ± 2.0	52.7 ± 4.6	14 ± 1.2	144.7 ± 14.9	10.4± 1.3	5.4 ± 1.3	17.6 ± 2.21
14	36.6 ± 2.3	64 ± 5.3	18.2 ± 1.5	144.7 ± 14.9	9.9 ± 1.4	5.4 ± 1.2	22 ± 2.6
15	47 ± 2.7	94.2 ± 6.8	19 ± 1.5	144.7 ± 14.9	18 ± 2.1	8 ± 1.6	51.9 ± 6.3
16	53.1 ± 3	100.5 ± 7.1	23.6 ± 1.7	155.7 ± 15.6	31.6 ± 3	11.4 ± 2.1	67.4 ± 7.5
17	57 ± 3.1	100.5 ± 7.1	28.1 ± 1.9	166.4 ± 16.3	40 ± 3.5	18.2 ± 2.9	74.1 ± 7.9
18	64.5 ± 3.4	110.6 ± 8.3	33.4 ± 2.2	178.5 ± 17.1	49.5 ± 4.5	20.8 ± 3.2	97.3 ± 9.5
19	72.5 ± 3.7	125 ± 9	36.9 ± 2.7	190.1 ± 17.9	65.9 ± 5.4	38.6 ± 5.1	103.8 ± 9.9
20	74.1 ± 3.7	125 ± 9	39 ± 2.8	203.3 ± 18.7	75.2 ± 5.9	47.8 ± 5.9	110.6 ± 10.4
21	82.3 ± 4.5	132.7 ± 9.4	46.7 ± 3.9	216 ± 19.4	105.2 ± 7.4	70.4 ± 7.7	148.9 ± 12.6
22	99.3 ± 5.1	157.5 ± 10.5	57.1 ± 4.4		123.5 ± 8.2	76.8 ± 8.1	185.2 ± 15.6
23	106.5 ± 5.4	157.5 ± 10.5	67.4 ± 5.5		136.6 ± 9.5	91.1 ± 9.1	195.1 ± 16.1

Relative tumour sizes (mean ± standard error) of tumours induced by fully tumourigenic, partially tumourigenic and combined total of the α-particle induced foci							
	(+)			(X/+)	(-)		
Incubation week	Total	Full	Partial	Full	Total	Full	Partial
24	135 ± 6.3	210.6 ± 12.7	79.5 ± 5.5		151.4 ± 10.2	107.2 ± 11.5	205.4 ± 16.7
25	143.1 ± 6.6	210.6 ± 12.7	91.1 ± 6.1		166.4 ± 10.9	125 ± 12.8	216 ± 17.3
26	145.5 ± 6.6	210.6 ± 12.7	95.4 ± 6.9		178.5 ± 11.4	144.7 ± 14.1	
27	158.3 ± 7.0	210.6 ± 12.7	115.5 ± 7.8		190.1 ± 11.9	166.4 ± 16.3	
28	167.3 ± 7.3	210.6 ± 12.7	129.6 ± 8.4		203.3 ± 12.4	190.1 ± 17.9	
29	177.5 ± 7.6	210.6 ± 12.7	147.2 ± 9.2		216 ± 13	216 ± 19.4	
30	179.4 ± 7.6	210.6 ± 12.7	151.4 ± 9.9				
31	196.1 ± 8.1	210.6 ± 12.7	182.3 ± 10.6				
32	216 ± 8.6	210.6 ± 12.7	216 ± 11.9				
33		216 ± 13					

Appendix 5.2.1: Mean tumour size (± standard error) of tumours induced by alpha-particle induced focus cells. The data for the foci were divided into that for fully tumourigenic foci and partially tumourigenic foci and combined to give the mean tumour size for all the tumourigenic foci. The data for the partially tumourigenic foci only include the tumours which actually grew (thus mice without tumours were excluded from the calculations). Data are also presented in figures 5.2.1. to 5.2.6. Relative tumour size is proportional to volume, using the formula r^3 where r represents the estimated radius of the tumours.

Relative tumour sizes (mean ± standard error) of tumours induced by fully tumourigenic, partially tumourigenic and combined total of the X-ray induced foci												
	(+) foci			(X/+) foci			(X) foci			(-)		
Incubation week	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial
1	<0.1 ± 0.1	<0.1 ± 0.1	0	0	0	0	0	0	0	0	0	0
2	<0.1 ± 0.1	<0.1 ± 0.1	0	<0.1 ± 0.1	<0.1 ± 0.1	0	0	0	0	0	0	0
3	<0.1 ± 0.1	0.1 ± 0.02	0	0.2 ± 0.1	<0.1 ± 0.1	1 ± 0.8	0	0	0	<0.1 ± 0.1	<0.1 ± 0.1	0
4	0.2 ± 0.1	0.6 ± 0.1	<0.1 ± 0.1	1 ± 0.4	0	8 ± 3	0	0	0	0.1 ± 0.1	0	1 ± 0.7
5	1.1 ± 0.2	5.1 ± 0.4	<0.1 ± 0.1	3.9 ± 1	<0.1 ± 0.1	27 ± 6.8	0	0	0	4.6 ± 1.1	<0.1 ± 0.1	34.3 ± 7.9
6	2 ± 0.3	13.5 ± 0.8	<0.1 ± 0.1	9.1 ± 1.7	0.1 ± 0.04	52.7 ± 10.5	0	0	0	9.7 ± 1.8	<0.1 ± 0.1	64 ± 12
7	5.6 ± 0.6	26.7 ± 1.6	0.2 ± 0.1	18.8 ± 3	1.2 ± 0.4	76.8 ± 13.5	0	0	0	21.3 ± 3	0.2 ± 0.1	125 ± 18.8
8	12.6 ± 1	44.4 ± 2.3	1.4 ± 0.4	39 ± 4.8	5.5 ± 1	125 ± 18.8	0	0	0	41.1 ± 4.6	1.5 ± 0.3	190 ± 24.8
9	18.2 ± 1.2	55.7 ± 2.6	3 ± 0.6	56.2 ± 6.2	18.6 ± 2.5	125 ± 18.8	0	0	0	63.5 ± 6.2	7.8 ± 1.1	216 ± 27
10	27.8 ± 1.7	77.9 ± 3.3	5.6 ± 1	75.7 ± 7.5	32.8 ± 4	144.7 ± 20.7	0	0	0	84 ± 7.5	21 ± 2.1	
11	33.7 ± 1.9	95.4 ± 3.8	6.8 ± 1.1	102.5 ± 9.2	57.1 ± 5.8	166.4 ± 22.7	0	0	0	103.2 ± 8.6	38.6 ± 3.4	
12	41.1 ± 2.1	117.6 ± 4.3	8 ± 1.2	124.3 ± 10.5	75.7 ± 7.5	190.1 ± 24.8	0.3 ± 0.4	2.4 ± 0.9	0	119.8 ± 9.5	57.1 ± 4.4	
13	44 ± 2.2	128.8 ± 4.6	8 ± 1.2	142.2 ± 11.4	86.9 ± 8.2	216 ± 27	3.4 ± 1.9	8 ± 2.9	1 ± 1.5	132.7 ± 10.7	73.6 ± 5.3	
14	54 ± 4.3	143.1 ± 4.9	12.6 ± 3.1	176.6 ± 14.2	142.2 ± 12.3		6.2 ± 2.8	19 ± 5.1	1 ± 1.5	144.7 ± 10.7	91.1 ± 6.1	
15	58.4 ± 4.5	159.2 ± 5.3	12.6 ± 3.1	191.1 ± 14.9	168.2 ± 13.7		12.8 ± 4.6	19 ± 5.1	8 ± 6	144.7 ± 10.7	91.1 ± 6.1	
16	68.4 ± 5	166.4 ± 5.4	19 ± 4.1	203.3 ± 15.6	191.1 ± 14.9		27 ± 7.6	27 ± 6.5	27 ± 13.5	144.7 ± 10.7	91.1 ± 6.1	
17	79 ± 5.5	172.8 ± 5.6	27 ± 5.1	216 ± 16.2	216 ± 16.2		42.9 ± 10.3	27 ± 6.5	64 ± 24	147.2 ± 10.9	94.8 ± 6.2	

Relative tumour sizes (mean ± standard error) of tumours induced by fully tumourigenic, partially tumourigenic and combined total of the X-ray induced foci												
	(+) foci			(X/+) foci			(X) foci			(-)		
Incubation week	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial
18	91.1 ± 6.1	182.3 ±5.8	36.9 ± 6.3				81.7 ± 16.4	49.4 ± 11.7	125 ± 37.5	144.7 ± 10.7	91.1 ± 6.1	
19	93 ± 6.2	188.1 ± 5.9	36.9 ± 6.3				152.3 ± 24.8	101.8 ± 19	216 ± 54	144.7 ± 10.7	91.1 ± 6.1	
20	116.2 ± 7.1	190.1 ± 6	64 ± 9.1				152.3 ± 24.8	101.8 ± 19		144.7 ± 10.7	91.1 ± 6.1	
21	116.2 ± 7.1	190.1 ± 6	64 ± 9.1				152.3 ± 24.8	101.8 ± 19		144.7 ± 10.7	91.1 ± 6.1	
22	116.2 ± 7.1	190.1 ± 6	64 ± 9.1				182.3 ± 28	151.4 ± 24.7		147.2 ± 10.9	91.1 ± 6.1	
23	118.2 ± 7.2	196.1 ± 6.1	64 ± 9.1				216 ± 31.3	216 ± 31.3		150.6 ± 11.9	94.8 ± 6.2	
24	120.6 ± 7.3	203.3 ± 6.2	64 ± 9.1							150.6 ± 11.9	99.3 ± 7.1	
25	147.2 ± 10.9	203.3 ± 6.2	101.8 ± 16.4							153.1 ± 12	99.3 ± 6.4	
26	147.2 ± 10.9	203.3 ± 6.2	101.8 ± 16.4							161 ± 12.4	103.2 ± 7.3	
27	176.6 ± 12.3	203.3 ± 6.2	151.4 ± 21.3							158.3 ± 12.3	116.2 ± 7.9	
28	176.6 ± 12.3	203.3 ± 6.2	151.4 ± 21.3							158.3 ± 12.3	116.2 ± 7.9	
29	212.8 ± 13.9	209.6 ± 7.4	216 ± 27							161 ± 12.4	117.6 ± 7.9	
30	216 ± 14	216 ± 7.6								181.3 ± 13.5	118.4 ± 8	
31										185.2 ± 13.6	149.7 ± 9.3	
32										190.3 ± 13.9	149.7 ± 9.3	
33										216 ± 15.1	166.4 ± 10	
34											216 ± 13	

Appendix 5.2.2: Mean tumour size (± standard error) of tumours induced by X-ray induced focus cells.Table presents data on the mean tumour size (± standard error) each week after α-particle induced focus cells were injected. The data for the foci were divided into that for fully tumourigenic foci and partially tumourigenic foci and combined to give the mean tumour size for all the tumourigenic foci. The data for the partially tumourigenic foci only includes the tumours which actually grew (thus mice without tumours were excluded from the calculations). Data are also presented in figures 5.2.1. to 5.2.6. Relative tumour size is proportional to volume, using the formula r^3 where r represents the estimated radius of the tumours.

Relative tumour sizes (mean ± standard error) of tumours induced by fully tumourigenic, partially tumourigenic and combined total of tumourigenic foci: Spontaneous focus and the combined data of α-particle and X-ray data

	(+) foci			(X/+) foci			(X) foci			(-)			Spontaneous (+)
Incubation week	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial	Full
1	0	<0.1 ± 0.1	0	0	0	0	0	0	0	0	0	0	0
2	<0.1 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1	0	0	0	0	0	0	0	<0.1	0
3	<0.1 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1	0.2 ± 0.1	0	1 ± 0.8	0	0	0	0	0	<0.1	0
4	<0.1 ± 0.1	<0.1 ± 0.1	<0.1 ± 0.1	1 ± 0.2	0	8 ± 3	0	0	0	<0.1 ± 0.1	0	0.1 ± 0.1	0.2 ± 0.2
5	0.3 ± 0.04	0.8 ± 0.1	<0.1 ± 0.1	4.1 ± 0.6	<0.1 ± 0.1	27 ± 6.8	0	0	0	0.6 ± 0.1	0	4.3 ± 1.0	0.2 ± 0.2
6	0.6 ± 0.09	2.2 ± 0.2	<0.1 ± 0.1	9.3 ± 1.1	0.1 ± 0.04	52.7 ± 10.6	0	0	0	1.3 ± 0.2	<0.1 ± 0.1	8.5 ± 1.6	1 ± 0.6
7	2 ± 0.19	5.5 ± 0.5	0.4 ± 0.1	19.9 ± 2.2	1.6 ± 0.4	76.8 ± 13.5	0	0	0	3.2 ± 0.5	<0.1 ± 0.1	17.6 ± 2.6	4.1 ± 1.7
8	4.33 ± 0.32	9.9 ± 0.6	1.3 ± 0.2	37.6 ± 3.4	4.9 ± 0.8	125 ± 18.8	0	0	0	7.6 ± 0.9	0.7 ± 0.2	28.1 ± 3.6	10.6 ± 3.2
9	9.67 ± 0.54	21.7 ± 1.4	3.2 ± 0.4	54.4 ± 4.3	17.2 ± 2	125 ± 18.8	0	0	0	13.5 ± 1.4	3.3 ± 0.5	35 ± 4.2	27 ± 5.9
10	15.8 ± 0.76	31 ± 1.8	6.7 ± 0.6	83.5 ± 6.3	42.1 ± 4	144.7 ± 20.7	0	0	0	20.3 ± 1.8	7.2 ± 0.9	43.6 ± 5.2	46.7 ± 8.6
11	22.2 ± 0.95	51.1 ± 2.5	6.9 ± 0.7	117.6 ± 7.9	79.5 ± 6.1	166.4 ± 22.7	0	0	0	25.2 ± 2.1	10.5 ± 1	49.4 ± 5.7	97.3 ± 14

Relative tumour sizes (mean ± standard error) of tumours induced by fully tumourigenic, partially tumourigenic and combined total of tumourigenic foci: Spontaneous focus and the combined data of α-particle and X-ray data

	(+) foci			(X/+) foci			(X) foci			(-)			Spontaneous (+)
Incubation week	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial	Full
12	29.8 ± 1.15	68.9 ± 3	9.1 ± 0.8	139.8 ± 8.9	98.6 ± 7	190.1 ± 24.8	0.3 ± 0.3	2.4 ± 0.9	0	37.6 ± 2.7	19.1 ± 1.7	65 ± 6.8	175.6 ± 20.7
13	36.3 ± 1.64	85.2 ± 3.5	10.9 ± 0.96	159.2 ± 9.7	113.4 ± 7.7	216 ± 27	3.4 ± 1.9	8 ± 2.9	1 ± 1.5	48.2 ± 3.2	26.2 ± 2.4	79.5 ± 7.8	175.6 ± 20.7
14	44.7 ± 2.27	98.6 ± 3.8	15.3 ± 1.9	177.5 ± 9.5	143.9 ± 8.2		6.2 ± 2.8	19 ± 5.1	1 ± 1.5	50.7 ± 3.3	27 ± 2.2	85.2 ± 8.1	175.6 ± 20.7
15	52.7 ± 2.5	124.3 ± 4.5	15.6 ± 1.9	185.2 ± 9.7	156.6 ± 8.7		12.8 ± 4.6	19 ± 5.1	8 ± 6	61.2 ± 3.7	27 ± 2.2	115.5 ± 10.7	216 ± 23.8
16	60.7 ± 2.8	131.1 ± 4.7	21.3 ± 2.3	194.1 ± 10.1	172.8 ± 9.3		27 ± 7.6	27 ± 6.5	27 ± 13.5	74.6 ± 4.3	39 ± 3.1	128 ± 11.4	
17	67.4 ± 2.98	133.4 ± 4.7	27.5 ± 2.7	203.3 ± 10.4	190.1 ± 9.9		42.9 ± 10.3	27 ± 6.5	64 ± 24	82.3 ± 4.5	46.7 ± 3.5	132.7 ± 11.7	
18	77.3 ± 3.3	143.9 ± 5.8	35.3 ± 3.2	206.4 ± 10.5	197.1 ± 10.2		81.8 ± 16.4	49 ± 11.7	125 ± 37.5	89.3 ± 5.4	47.8 ± 3.6	148.9 ± 12.6	
19	82.3 ± 3.4	154.9 ± 6.1	36.9 ± 3.3	209.6 ± 10.6	203.3 ± 10.4		152.3 ± 24.8	101 ± 19	216 ± 54	100.5 ± 5.8	61.2 ± 4.2	153.1 ± 12.9	
20	93.6 ± 3.7	155.8 ± 6.1	50.7 ± 4.1	212.8 ± 10.7	209.6 ± 10.6		152.3 ± 24.8	101 ± 19		106.5 ± 6.1	67.4 ± 4.5	157.5 ± 13.1	
21	98.6 ± 3.8	160.1 ± 6.2	54.9 ± 4.8	216 ± 10.8	216 ± 10.8		152.3 ± 24.8	101 ± 19		124.3 ± 6.7	80.6 ± 5.0	180.1 ± 14.4	
22	107.9 ± 4.1	173.7 ± 6.5	60.7 ± 5.1				182.3 ± 28	151.4 ± 24.7		134.2 ± 7.1	84 ± 5.2	200.2 ± 15.4	

Relative tumour sizes (mean ± standard error) of tumours induced by fully tumourigenic, partially tumourigenic and combined total of tumourigenic foci: Spontaneous focus and the combined data of α-particle and X-ray data

	(+) foci			(X/+) foci			(X) foci			(-)			Spontaneous (+)
Incubation week	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial	Full
23	112.7 ± 4.2	176.6 ± 6.6	65.9 ± 5.4				216 ± 31.2	216 ± 31.3		142.2 ± 7.4	93 ± 5.5	205.4 ± 15.7	
24	128 ± 4.6	207.5 ± 7.4	71.5 ± 5.7							151.4 ± 7.7	103.2 ± 6.6	210.6 ± 15.9	
25	145.5 ± 6.64	207.5 ± 7.4	96.7 ± 7							158.3 ± 7.9	112 ± 7	216 ± 16.2	
26	146.4 ± 6.7	207.5 ± 7.4	98.6 ± 9							165.5 ± 8.1	123 ± 7.4		
27	167 ± 7.3	207.5 ± 7.4	132.7 ± 10.9							175.6 ± 8.5	140 ± 8.1		
28	171 ± 7.4	207.5 ± 7.4	140.6 ± 11.4							180.4 ± 8.6	147 ± 8.4		
29	195.1 ± 8.1	210.6 ± 7.4	179.4 ± 13.4							186.2 ± 8.8	158 ± 8.8		
30	197.1 ± 8.1	213.8 ± 7.5	182.3 ± 13.5							187.1 ± 8.8	161 ± 8.9		
31	206.4 ± 8.4	213.8 ± 7.5	199.2 ± 14.3							198.2 ± 9.2	181 ± 9.0		
32	216 ± 8.6	216 ± 7.8	216 ± 15							200.2 ± 9.2	185 ± 9.3		
33										203.3 ± 9.3	190 ± 9.9		

Relative tumour sizes (mean ± standard error) of tumours induced by fully tumourigenic, partially tumourigenic and combined total of tumourigenic foci: Spontaneous focus and the combined data of α-particle and X-ray data													
	(+) foci			(X/+) foci			(X) foci			(-)			Spontaneous (+)
Incubation week	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial	Total	Full	Partial	Full
34										216 ± 9.7	216 ± 10.8		

Appendix 5.2.3: Mean tumour size (± standard error) of tumours induced by radiation-induced foci (combined data of alpha-particle and X-ray induced foci). Table presents data on the mean tumour size (± standard error) each week after radiation induced focus cells (combined data of α-particle and X-ray induced foci data) and the spontaneous focus cells were injected. The data for the foci were divided into that for fully tumourigenic foci and partially tumourigenic foci and combined to give the mean tumour size for all the tumourigenic foci. The data for the partially tumourigenic foci only include the tumours which actually grew (thus mice without tumours were excluded from the calculations). Data are also presented in figures 5.2.1. to 5.2.6. Relative tumour size is proportional to volume, using the formula r^3 where r represents the estimated radius of the tumours.

Section 5.3.

Results of Focus Reconstruction Studies

List of figures

The identification of transformed foci in the C3H10T½ transformation assay relies on the ability of the transformed cells to overcome routine growth regulatory controls so that they continue to grow forming the multilayers of cells which constitute foci. Furthermore the way in which cells within foci are arranged determines the subsequent classification of the focus. The aim of the studies presented in this section was to determine if these properties were retained by the focus cells after the cells had been isolated and expanded to produce a focus cell line, and also to examine if the same properties were carried through to the tumour cells isolated from focus-induced tumours in C3H mice. Thus a sample of foci from the various focus categories; (+), (X/+), (X), (-), induced by X-rays, alpha-particles and spontaneously were examined along with corresponding tumour cells (see figure 5.3.1 for a list of the cell lines examined).

Several authors have reported suppression of transformation (a reduction in the number of transformed foci observed) using a variety of methods including the treatment of cultures of C3H10T½ cells (previously exposed to carcinogens) with ascorbic acid (Benedict *et al.* 1980, Tauchi and Sawada 1993), retinoids (Lloyd *et al.* 1978, Merriman and Bertram 1979), or exposing the cells to the carcinogen at high cell density (Reznikoff *et al.* 1973, Haber *et al.* 1977, Bettega *et al.* 1989). Lloyd *et al.* (1978) reported that the expression of transformation could be completely suppressed by co-cultivating transformed cells with a large number of untransformed cells. A focus cell line developed from a type III C3H10T½ focus, induced by alpha-particle irradiation was used for the studies by Lloyd *et al.* They found that the suppression of the transformed focus appearances only occurred up to a certain number of untransformed cells, when the number of untransformed cells to transformed cells was increased further, a reversal to more prominent foci was observed in the cell cultures. Their conclusions were, that under some conditions, transformed cells could be made to alter their phenotypic expression to appear normal in culture by the addition of untransformed cells (Lloyd *et al.* 1978). The same effect was observed in studies using a type III focus induced by Benzo (A) pyrene (Lloyd *et al.* 1978).

The experiments presented in this chapter are similar in design to those described by Smith *et al.* (1993) where a focus-forming efficiency was calculated for

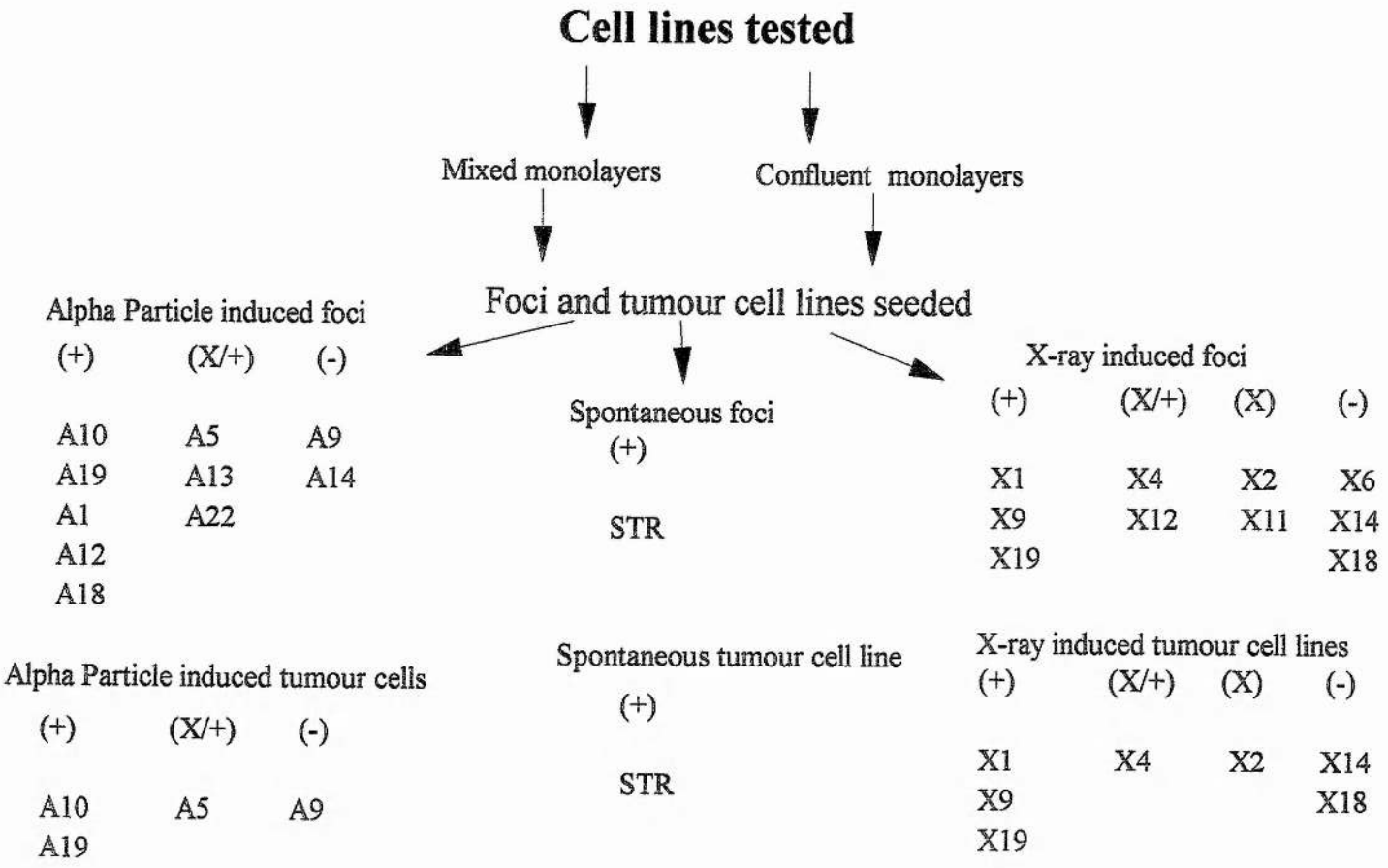


Figure 5.3.1. List of foci and tumour cells tested in the focus reconstruction studies. The prefix A denotes the alpha - particle induced foci / tumour cells and the prefix X denotes the X-ray induced foci / tumour cells. The cell lines tested were as described in table 5.1.2 and 5.1.3. All tumourigenic foci were examined with their corresponding tumour cell lines and foci listed above without a corresponding tumour cell line were non-tumourigenic.

focus cells seeded on to confluent monolayers of untransformed cells or seeded in suspension with equivalent numbers of untransformed cells (mixed monolayers). Smith's data found a strong correlation between the reconstruction of foci when seeded in suspension with untransformed cells and when seeded on to confluent monolayers. These data also indicated that the focus reconstruction studies were highly predictive of the tumourigenicity of the foci, even more so than the anchorage - independent growth assay which is widely regarded as a reliable indicator of tumourigenicity (Smith *et al.* 1993).

Mixed monolayers are quite similar to the monolayers which develop during a transformation assay where the transformed cells are attached to the culture dish surrounded by untransformed cells. Thus one expects a similar process to occur in the mixed monolayers as occurs in the transformation assay where once the cells reach confluence the transformed cells continue to divide and form a focus growing over the monolayer of untransformed cells. Focus reconstruction studies on confluent monolayers of untransformed cells remove the step(s) of the transformed and untransformed cells reaching confluence together and thus any suppression effect this may have since in this case the transformed cells are seeded on to monolayers which are already confluent.

Experiment details and calculation of focus frequencies

The details of the experiments are given in chapter two, however figure 5.3.2 illustrates the main points. One part of the experiment was a straightforward plating of the focus / tumour cells on to an established confluent monolayer of untransformed C3H10T½ cells. For the other part of the experiment, a cell suspension comprising a mixture of focus or tumour cells and untransformed cells were seeded, using the same cell numbers as for the first part.

At the end of the protocol for the focus reconstruction studies described in figure 5.3.2 the culture dishes were stained and examined for foci which were categorised as (+), (X/+), (X) or (-), as previously described in section 5.1. The number of foci on each culture dish was also recorded. A 'focus frequency' was calculated for all cell lines (focus / tumour) examined. This was calculated for all cell

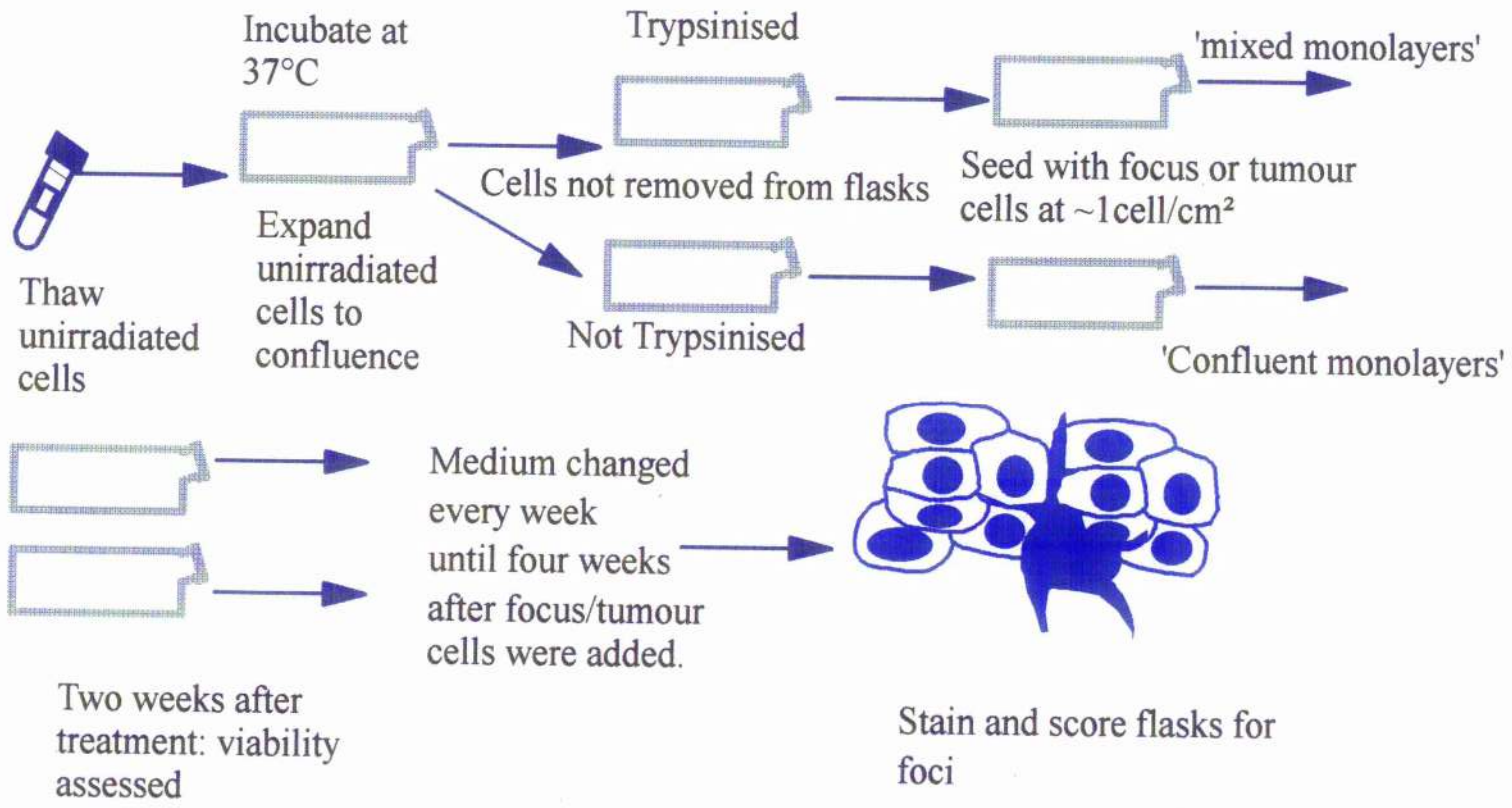


Figure 5.3.2. Outline of the procedure used for the focus reconstruction studies.

lines which produced foci (of any category) on the culture dishes and further calculations were done based on whether the foci produced were (+), (X/+), (X) or (-) foci. The frequency of foci produced by the cells was calculated in the same manner as widely used for the calculation of transformation frequency by carcinogens, using the equation shown in chapter three.

Sample calculation of focus frequency

Cell line P produces eight foci categorised as (+), (+), (X/+),(-) (X) on one culture and (X), (X), (X) on another culture in a total number of eight cultures seeded with P cells. 500 P cells are seeded per culture and the plating efficiency is calculated as 50%. Focus frequencies are calculated for (a) all categories of reconstructed foci produced, (b) reconstructed (+) foci, (c) reconstructed (X/+) foci, (d) reconstructed (X) foci and (e) reconstructed (-) foci produced.

(a) Focus frequency of all reconstructed foci produced:

F = Frequency of all reconstructed foci

D = Total number of culture flasks = 8

D_0 = Number of flasks without foci = 6

N = Total number of viable cells = Number of cells seeded multiplied by the plating efficiency = 500 x 50% = 250 viable cells per flask = 2000 viable cells (total)

$$F = \frac{-\ln\frac{6}{8}\times 8}{2000} \pm \frac{\left(\sqrt{\frac{-\ln\left(\frac{6}{8}\right)}{8}}\right)\times 8}{2000} = 1.15x10^{-3} \pm 0.75x10^{-3}$$

(b) Focus frequency of reconstructed (+) foci produced:

F = Frequency of reconstructed (+) foci

D = Total number of culture flasks = 8

D_0 = Number of flasks without (+) foci = 7

N = Total number of viable cells = 2000

$$F = \frac{-\ln\frac{7}{8}\times 8}{2000} \pm \frac{\left(\sqrt{\frac{-\ln\left(\frac{7}{8}\right)}{8}}\right)\times 8}{2000} = 5.34\times10^{-4} \pm 5.17\times10^{-4}$$

The frequencies of reconstructed (X/+) and (-) foci are the same as that for the (+) foci calculated above since like the (+) foci the (X/+) and (-) foci appear on only one culture whereas the frequency of reconstructed (X) foci is the same as calculated for all categories of reconstructed foci in the first sample calculation since reconstructed (X) foci appear on two cultures.

Glossary of phrases used in this section

A number of abbreviated phrases are used throughout this section to differentiate between the original foci from which cell lines were developed and the foci which were produced on the confluent and mixed monolayers. Sample phrases are outlined below:

'The (+) foci' translates as the cells derived from foci categorised as (+) in section 5.1.

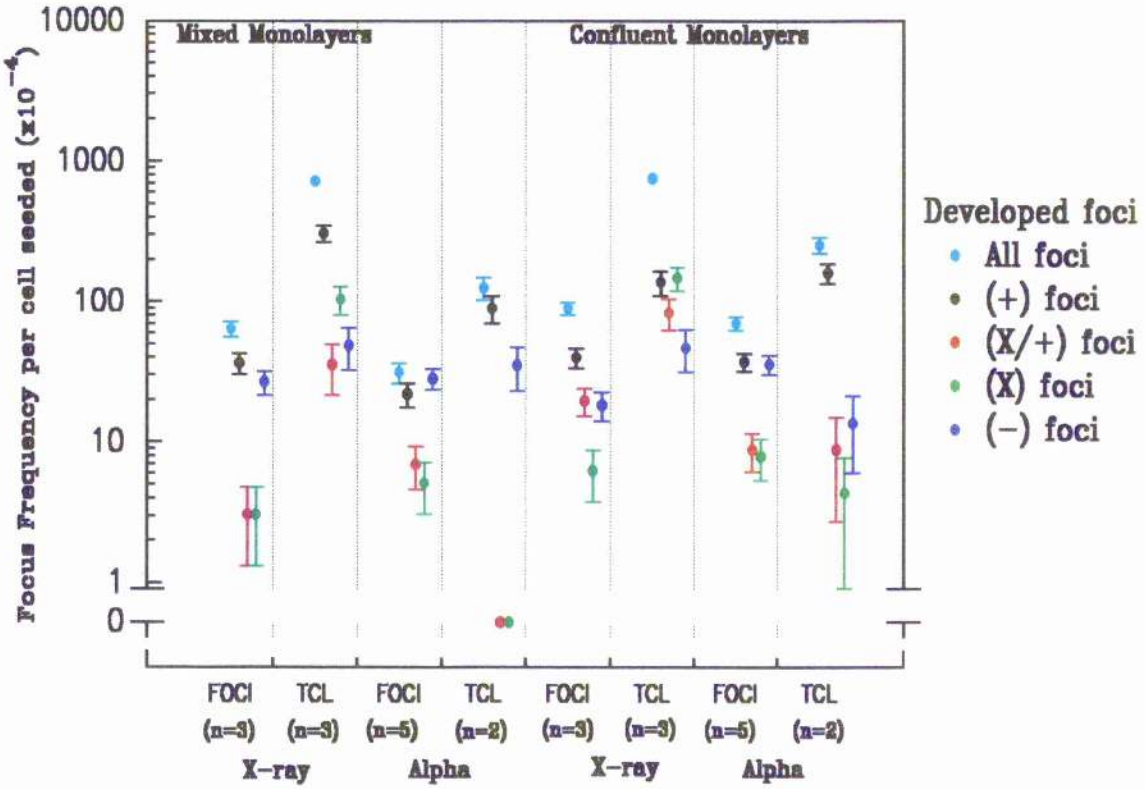
'The (+) tumour cells' translates as cells isolated from tumours induced by foci categorised as (+) in section 5.1.

'Reconstructed (+) foci on confluent monolayers' translates as foci which developed on the confluent monolayers and were categorised as (+) using the classification criteria presented in section 5.1. The word reconstructed appears in most instances to differentiate between the foci produced on the mixed or confluent monolayers and the original foci from which the focus and tumour cell lines were derived.

Focus frequency data: comparison of X-rays and alpha - particles for individual focus categories.

Figures 5.3.3 to 5.3.8 show the focus frequencies of all reconstructed foci produced, irrespective of category as well as those of the different types of reconstructed foci produced, by the various categories of foci and tumour cells seeded (X-ray, alpha - particle and spontaneously induced). In the following graphs the term 'all foci' refers to the frequency of focus - positive cultures produced per cell seeded irrespective of the type of reconstructed focus produced on those cultures. Figures 5.3.3 to 5.3.6 show the frequencies of reconstructed foci produced by the (+), (X/+), (X) and (-) cells respectively. Figures 5.3.7 and 5.3.8 show the same data as figures 5.3.3 to 5.3.6 presented to allow comparison of the different categories of foci or tumour cells.

Figure 5.3.3 (a) compares X-ray and alpha - particle induced (+) foci and tumour cells seeded on mixed and confluent monolayers of C3H10T½ cells. On both the mixed and confluent monolayers the X-ray induced (+) foci produced lower frequencies of all categories of reconstructed foci; (+), (X/+), (X) (-), than the corresponding tumour cells. The alpha - particle induced (+) foci produced lower frequencies of all categories of reconstructed foci (irrespective of focus category) and reconstructed (+) foci yet a higher frequency of reconstructed (X/+) and reconstructed (X) foci than the tumour cells on the mixed monolayers whereas on the confluent monolayers the frequency of all categories of reconstructed foci (irrespective of focus category) and reconstructed (+) foci is still lower while the frequency of reconstructed (-) foci is higher for the foci than for the tumour cells. Comparison of the foci showed little difference between X-rays and alpha - particles on the mixed monolayers although the X-ray induced foci showed a higher frequency of all categories of reconstructed foci (irrespective of focus category) and reconstructed (+) foci than the alpha - particle equivalent. On the confluent monolayers the X-ray induced (+) foci again showed a higher frequency of all categories of reconstructed foci (irrespective of focus category) while the frequency of reconstructed (X/+) was higher and that of reconstructed (-) foci was lower than produced by the alpha - particle induced (+) foci. Comparison of the tumour cells showed greater frequencies of all categories of



(+) cells seeded

Figure 5.3.3 (a). Frequency of reconstructed foci produced per (+) focus / tumour cell seeded in monolayers of C3H10T½ cells. The mixed monolayers were produced by seeding a cell suspension comprising a mixture of untransformed C3H10T½ cells with the (+) focus/ tumour cells of interest (n = number of cell lines examined in each category). Culture dishes were examined for the presence of (+), (X/+), (X) and (-) foci. TCL is the abbreviation for tumour cell line(s).

reconstructed foci (irrespective of focus category), (+), (X/+) and (X) foci were produced by the tumour cells from the X-ray induced foci than the corresponding alpha - particle equivalent on the mixed monolayers. The same observations were noted on the confluent monolayers, this time including a higher frequency of reconstructed (-) foci rather than reconstructed (+) foci produced by the tumour cells from the X-ray induced foci. Comparison of the focus frequencies on the mixed and confluent monolayers revealed similar patterns for the focus cells (X-ray and alpha-particle induced) with a higher frequency of reconstructed (X/+) foci observed on the confluent monolayers for the X-ray induced foci. The tumour cells from the X-ray induced (+) foci had a lower frequency of reconstructed (+) foci and a higher frequency of reconstructed (X/+) foci while the tumour cells from the alpha - particle induced foci showed a lower frequency of reconstructed (-) foci and higher frequencies of reconstructed (X/+) and (X) foci on the confluent monolayers compared to the mixed monolayers.

Figure 5.3.3 (b) compares the frequency of reconstructed foci produced by the spontaneous focus and tumour cells on mixed and confluent monolayers of C3H10T½ cells. Tumour cells produced higher frequencies of all categories of reconstructed foci (irrespective of focus category), (X/+), (X) and (-) foci on the mixed monolayers than the corresponding original foci seeded. The differences noted on the confluent monolayers were a higher frequency of reconstructed (-) foci and lower frequencies of reconstructed (X/+) and (X) foci produced by the spontaneous focus compared to the tumour cell line. Comparison of the mixed and confluent monolayers showed no significant differences between the foci except a slightly lower frequency of reconstructed (-) foci produced on the confluent monolayers. A similar comparison for the tumour cells presented a higher frequency of reconstructed (-) foci and a lower frequency of reconstructed (X) foci on the mixed monolayers compared to the confluent monolayers.

Figure 5.3.4 displays the frequency of reconstructed foci produced by (X/+) foci and their corresponding tumour cells seeded on confluent and mixed monolayers of C3H10T½ cells. The X-ray induced (X/+) focus cells seeded on both mixed and confluent monolayers showed a lower focus frequency of all reconstructed foci

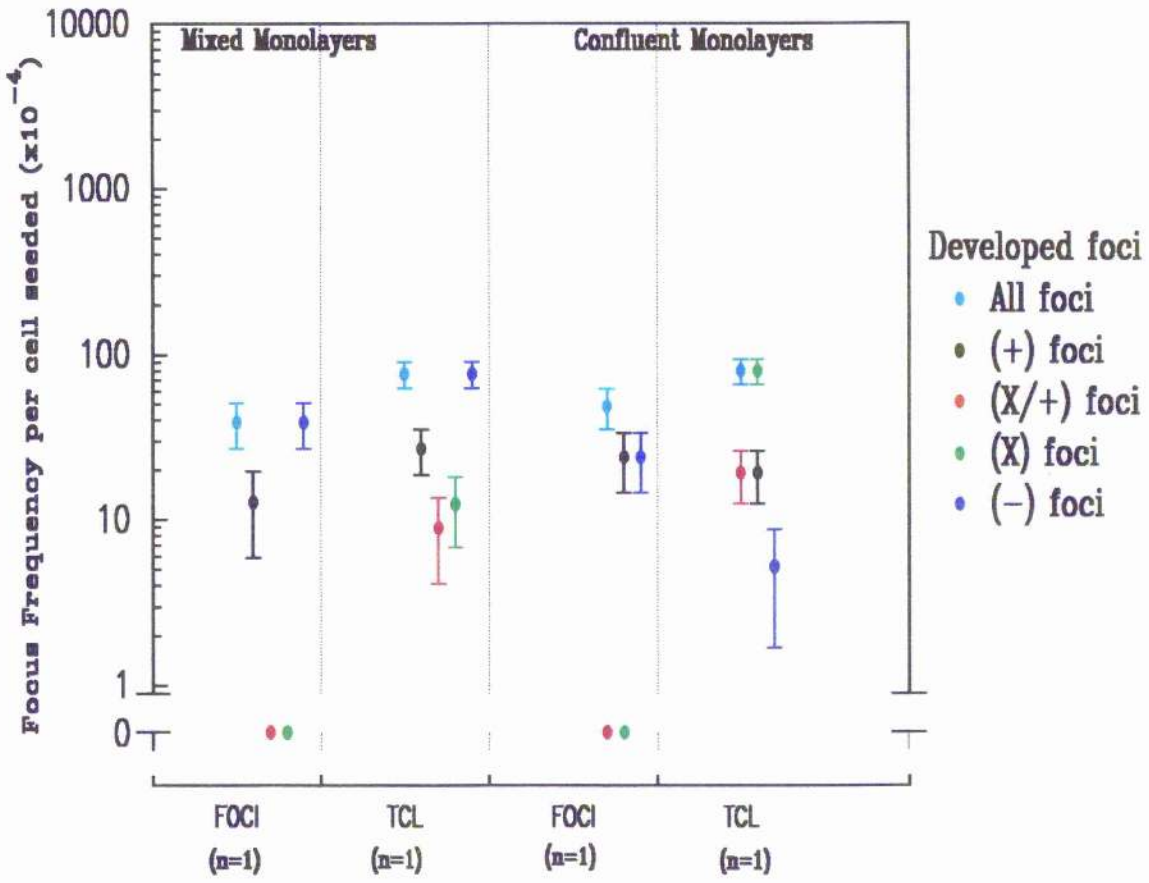


Figure 5.3.3 (b). Frequency of reconstructed foci produced per (+) focus / tumour cell seeded in monolayers of C3H10T½ cells. The mixed monolayers were produced by seeding a cell suspension comprising a mixture of untransformed C3H10T½ cells with the (+) focus/ tumour cells of interest (n = number of cell lines examined in each category). Culture dishes were examined for the presence of (+), (X/+), (X) and (-) foci. TCL is the abbreviation for tumour cell line(s)

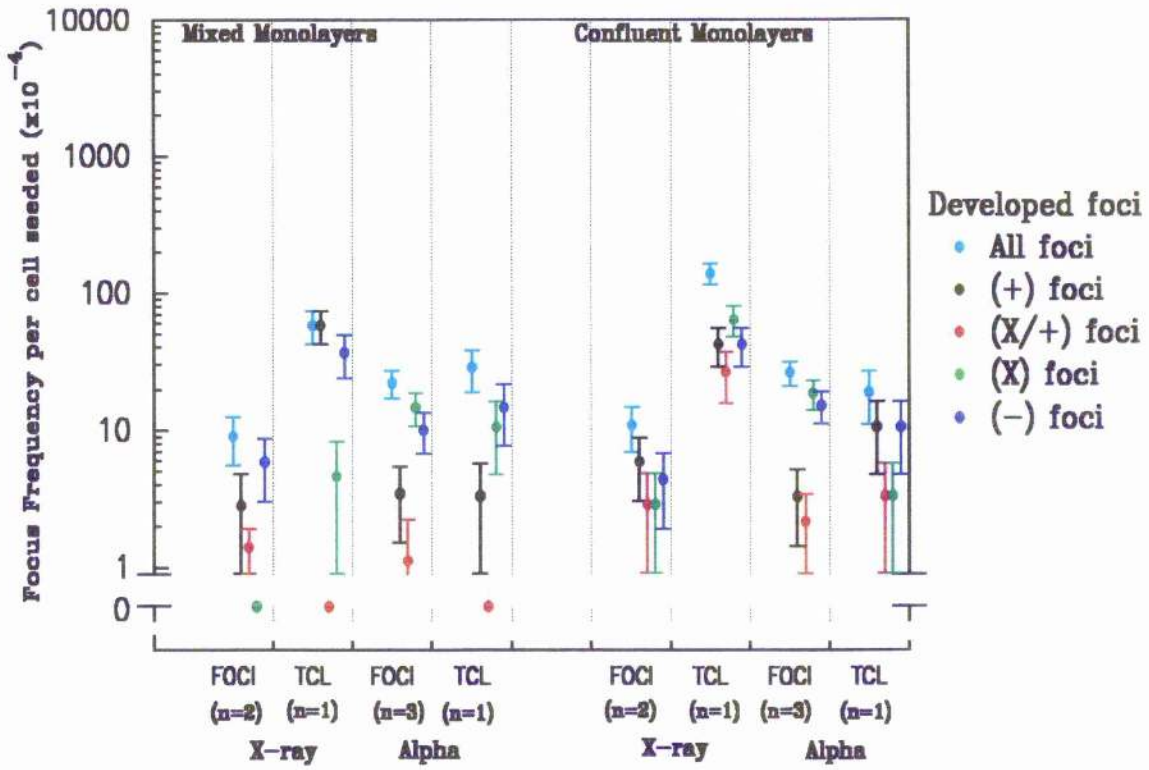
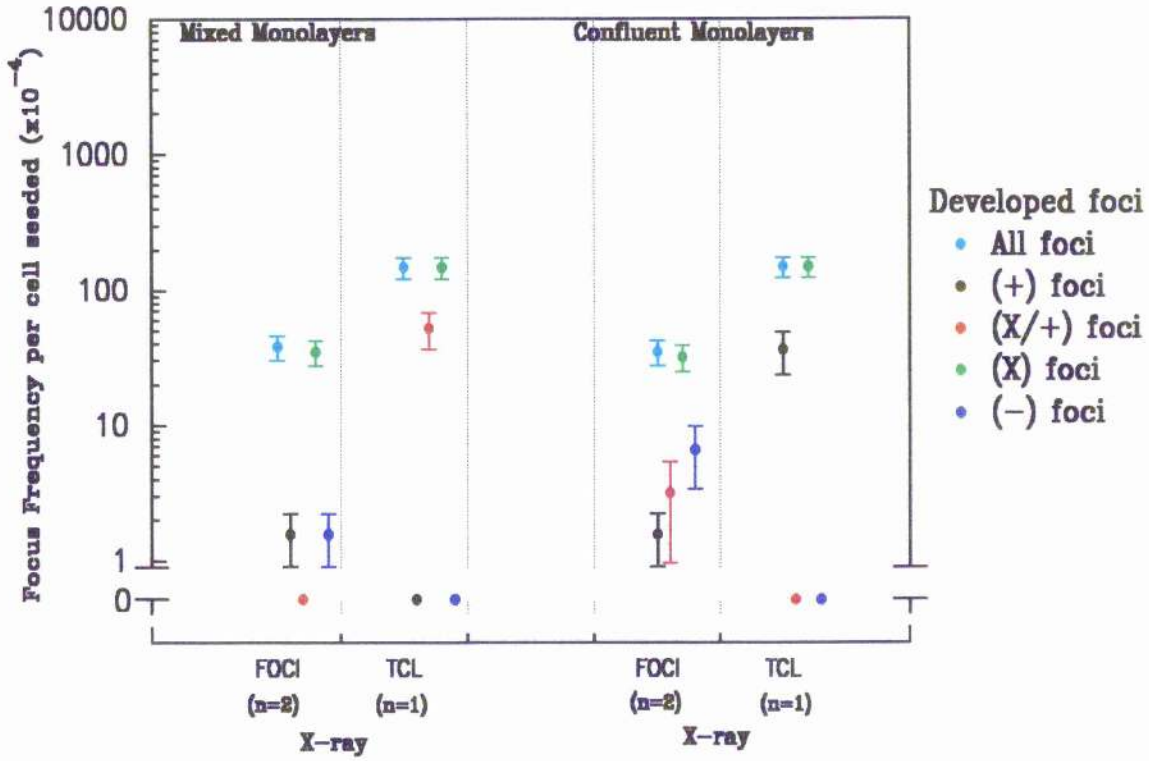


Figure 5.3.4. Frequency of reconstructed foci produced per (X/+) focus / tumour cell seeded in monolayers of C3H10T½ cells. The mixed monolayers were produced by seeding a cell suspension comprising a mixture of untransformed C3H10T½ cells with the (X/+) focus/ tumour cells of interest (n = number of cell lines examined in each category). Culture dishes were examined for the presence of (+), (X/+), (X) and (-) foci. TCL is the abbreviation for tumour cell line(s)

(irrespective of focus category) than the corresponding tumour cells. Furthermore the X-ray induced (X/+) foci showed lower frequencies of reconstructed (+), (X) and (-) foci on both mixed and confluent monolayers than the corresponding tumour cells and a lower frequency of reconstructed (X/+) foci on the confluent monolayers only. The alpha - particle induced (X/+) foci and tumour cells showed few differences on either mixed or confluent monolayers with the exception of an increased frequency (relative to the tumour cells) of reconstructed (X) foci produced by the original foci on the confluent monolayers. Comparison of the foci shows the alpha - particle induced foci produced higher frequencies of all categories of reconstructed foci (irrespective of focus category) and reconstructed (X) foci than the X-ray induced foci on both the mixed and confluent monolayers and a higher frequency of reconstructed (-) foci on the confluent monolayers. Comparison of the tumour cells shows the tumour cells from the X-ray induced foci produced higher frequencies of all categories of reconstructed foci, (+), (X/+), (X), and (-) on the confluent monolayers and higher frequencies of all categories of reconstructed foci (irrespective of focus category), (+) and (-) foci on the mixed monolayers. Comparison of the focus frequencies on the mixed and confluent monolayers revealed similar patterns for the focus cells seeded, with some differences apparent in the pattern of focus frequencies from the tumour cells seeded. Tumour cells from the X-ray induced (X/+) foci had higher frequencies of all reconstructed foci (irrespective of focus category), (X) and (X/+) foci on the confluent monolayers.

Figure 5.3.5 displays the frequency of reconstructed foci produced by X-ray induced (X) foci and their corresponding tumour cells seeded on confluent and mixed monolayers of C3H10T½ cells. The tumour cells displayed higher frequencies of all reconstructed foci (irrespective of focus category) and of reconstructed (X) and (X/+) foci and a lower frequency of reconstructed (-) foci on the mixed monolayers than the corresponding foci. Higher frequencies of all reconstructed foci (irrespective of focus category) and of reconstructed (X) and (+) foci and lower frequencies of reconstructed (X/+) and (-) foci were produced on the confluent monolayers by the tumour cells compared to the corresponding focus cells. The pattern of reconstructed foci developed by the focus cells was similar on both the mixed and confluent monolayers. The

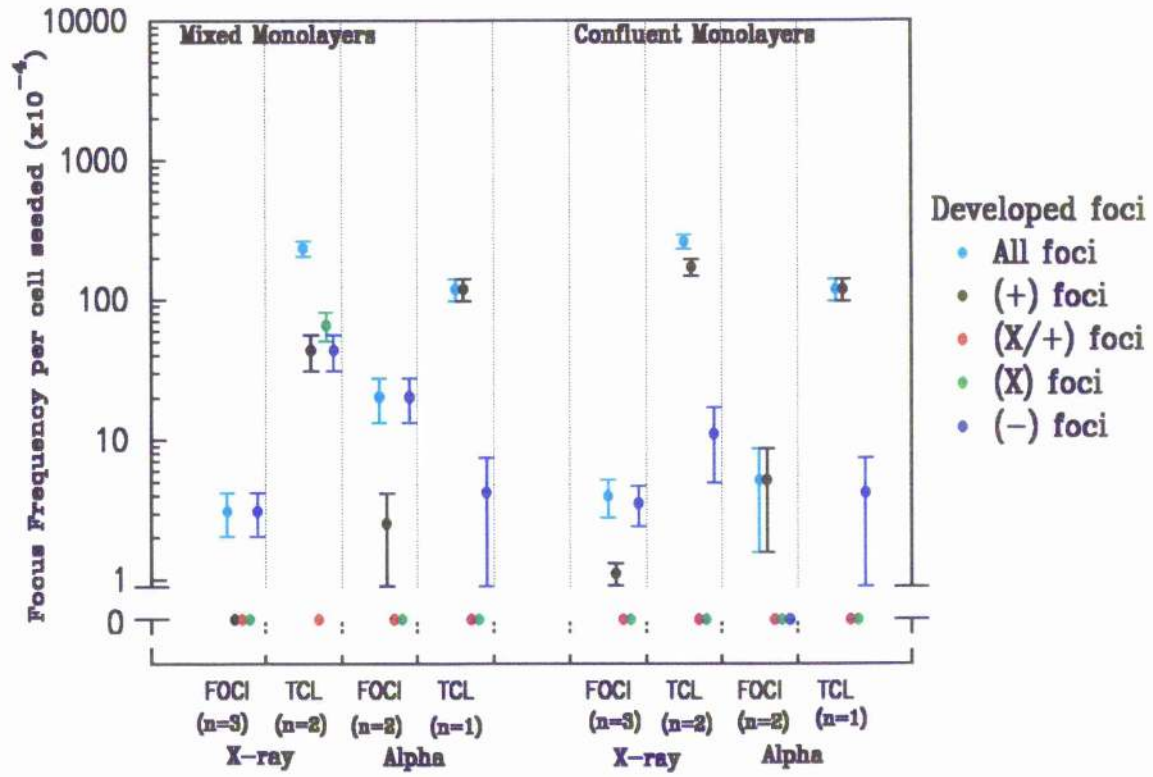


(X) cells seeded

Figure 5.3.5. Frequency of reconstructed foci produced per (X) focus / tumour cell seeded in monolayers of C3H10T½ cells. The mixed monolayers were produced by seeding a cell suspension comprising a mixture of untransformed C3H10T½ cells with the (X) focus/ tumour cells of interest (n = number of cell lines examined in each category). Culture dishes were examined for the presence of (+), (X/+), (X) and (-) foci. TCL is the abbreviation for tumour cell line(s)

tumour cell pattern was also similar for both mixed and confluent monolayers except for a higher frequency of reconstructed (X/+) on the mixed monolayers and of reconstructed (+) foci on the confluent monolayers.

Figure 5.3.6 displays the frequency of reconstructed foci produced by (-) foci and their corresponding tumour cells seeded on confluent and mixed monolayers of C3H10T½ cells. Tumour cells from the X-ray induced foci produced higher frequencies of all categories of reconstructed foci except the (X/+) foci on the mixed monolayers, while on the confluent monolayers the tumour cells produced higher frequencies of all reconstructed foci (irrespective of focus category) and of reconstructed (+) foci compared to the original focus cells examined. Tumour cells from the alpha - particle induced foci produced a higher frequency of reconstructed (+) foci on both types of monolayers and a lower frequency of reconstructed (-) foci on the mixed monolayers compared to the foci. Comparison of the foci shows that the alpha - particles produced higher frequencies of all categories of reconstructed foci (irrespective of focus category) and of reconstructed (+) and (-) foci than the X-rays on the mixed monolayers while the reverse was true for the frequency of reconstructed (-) foci on the confluent monolayers. Comparison of the tumour cells showed the X-rays produced higher frequencies of all categories of reconstructed foci (irrespective of focus category), (X) and (-) foci and a lower frequency of reconstructed (+) foci than the alpha - particles on the mixed monolayers while only higher frequencies of all categories of reconstructed foci (irrespective of focus category) and reconstructed (+) foci were observed on the confluent monolayers. Comparison of the mixed and confluent monolayers showed little difference for the X-ray induced foci (higher frequency of reconstructed (+) foci on the confluent monolayers) or the tumour cells from the alpha - particle induced foci. Tumour cells from the X-ray induced foci showed higher frequencies of reconstructed (X) and (-) foci and lower frequencies of reconstructed (+) foci on the mixed monolayers compared to the confluent monolayers. The alpha - particle induced foci showed higher frequencies of all categories of reconstructed foci (irrespective of focus category) and of reconstructed (-) foci on the mixed versus confluent monolayers.



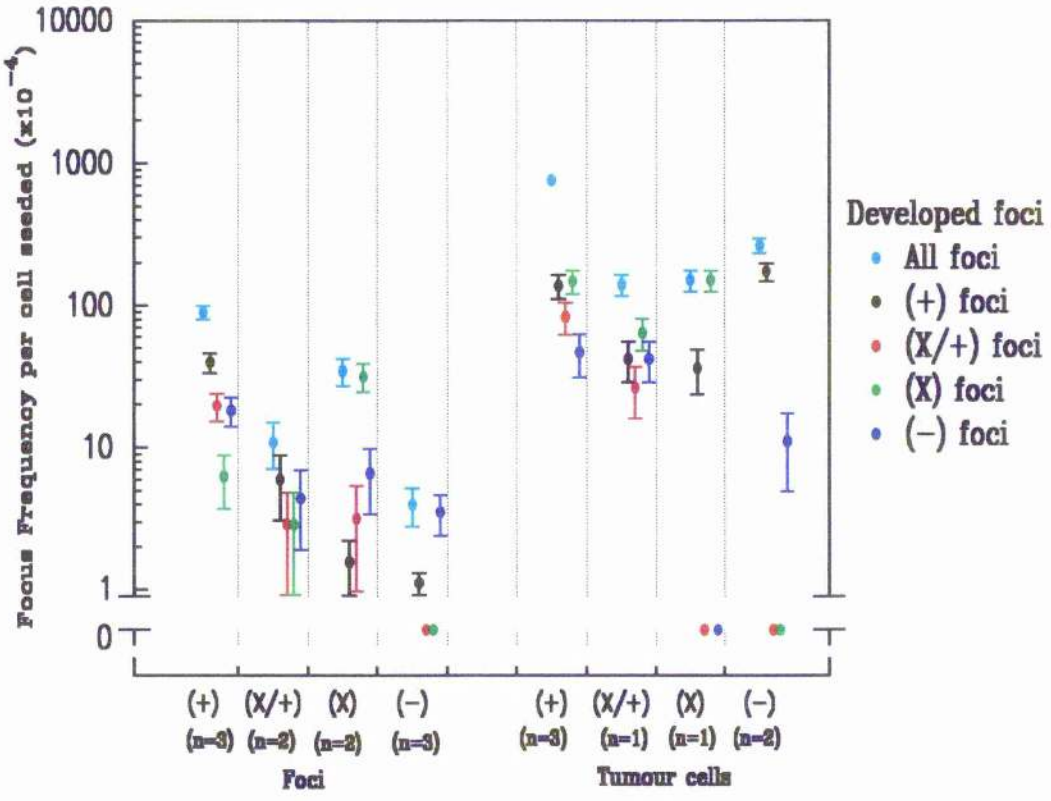
(-) cells seeded

Figure 5.3.6. Frequency of reconstructed foci produced per (-) focus / tumour cell seeded in monolayers of C3H10T½ cells. The mixed monolayers were produced by seeding a cell suspension comprising a mixture of untransformed C3H10T½ cells with the (-) focus/ tumour cells of interest (n = number of cell lines examined in each category). Culture dishes were examined for the presence of (+), (X/+), (X) and (-) foci. TCL is the abbreviation for tumour cell line(s)

Focus frequency data: comparison of focus categories for X-rays and alpha - particles.

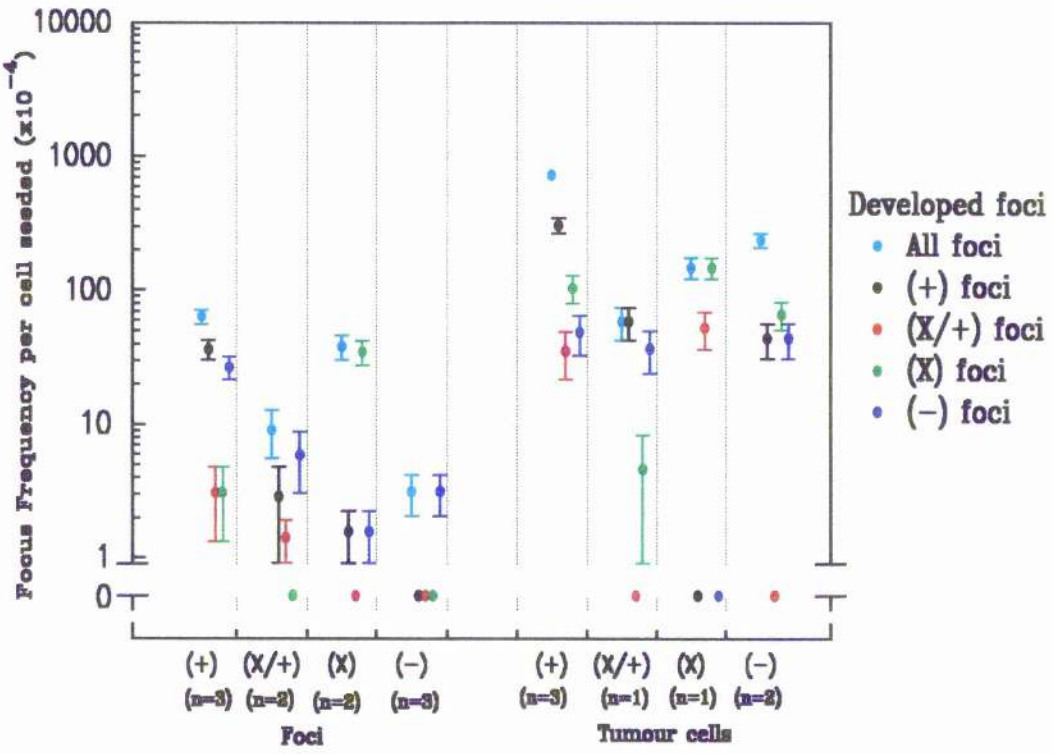
Figure 5.3.7 (a) illustrates the frequency of reconstructed foci produced by X-ray induced foci and their corresponding tumour cells on confluent monolayers of C3H10T½ cells. Cells derived from the (+) and (X/+) foci produced lower frequencies of all categories of reconstructed foci than the corresponding tumour cells. Cells derived from (X) foci had lower frequencies of reconstructed (X) and (+) foci and higher frequencies of reconstructed (X/+) and (-) foci compared to the corresponding tumour cells. (-) foci produced a lower frequency of reconstructed (+) foci and similar frequencies of all other categories of reconstructed foci compared to the tumour cells. Examination of the focus frequencies of the various categories of X-ray induced foci revealed that the (+) foci produced the highest frequency of all reconstructed foci (irrespective of focus category) and the highest frequency of reconstructed (+), (X/+), and (-) foci, while the highest frequency of reconstructed (X) foci was produced by cells from the (X) foci examined. Examination of the focus frequencies of the tumour cells from the various categories of X-ray induced foci revealed that the (+) tumour cells produced the highest frequency of all reconstructed foci (irrespective of focus category), the highest frequency of reconstructed (+) foci was produced by the (-) tumour cells (with a slightly lower value produced by the (+) tumour cells), the highest frequency of reconstructed (X/+) foci was observed for the (+) tumour cells, while the highest frequency of reconstructed (X) foci was shared by (+) and (X) tumour cells and lastly the highest frequency of reconstructed (-) foci developed was shared by the (+) and (X/+) tumour cells.

Figure 5.3.7 (b) illustrates the frequency of reconstructed foci produced by X-ray induced foci and their corresponding tumour cells on mixed monolayers of C3H10T½ cells. The (+) foci had a lower frequency of all reconstructed foci than the corresponding tumour cells. Tumour cells from the (X/+) foci had higher frequencies of reconstructed (+), (X) and (-) foci while the foci had a higher frequency of reconstructed (X/+) foci. Tumour cells from the (X) foci had increased frequencies of reconstructed (X/+) and (X) foci and lower frequencies of reconstructed (+) and (-) foci than the original foci. Foci and tumour cells of the (-) focus category had similar frequencies of only the reconstructed (X/+) foci and the tumour cells displayed higher



X-ray induced foci and tumour cells
Confluent monolayers

Figure 5.3.7 (a). Frequency of reconstructed foci produced by cells isolated from X-ray induced foci and corresponding tumours when the focus / tumour cells were seeded on to confluent monolayers of C3H10T½ cells (n = number of cell lines examined in each category). Culture dishes were examined for the presence of (+), (X/+), (X) and (-) foci.



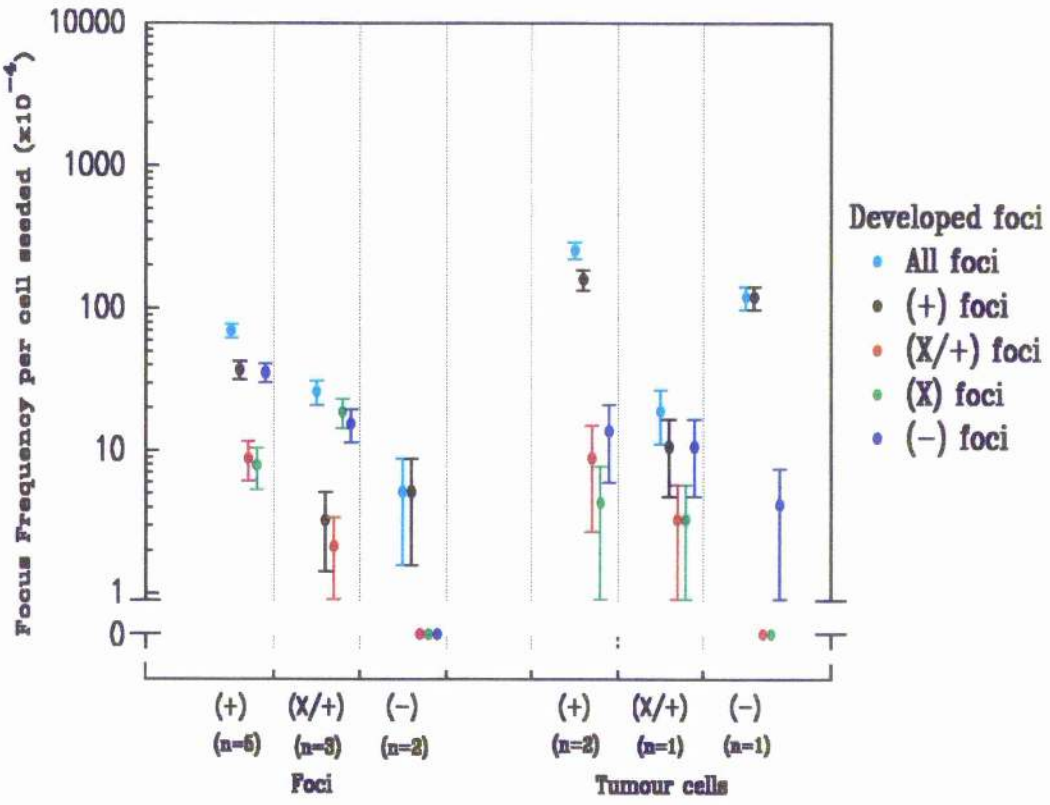
X-ray induced foci and tumour cells
Mixed monolayers

Figure 5.3.7 (b). Frequency of reconstructed foci produced by cells isolated from X-ray induced foci and corresponding tumours when the focus / tumour cells were seeded in monolayers of C3H10T½ cells (n = number of cell lines examined in each category). The mixed monolayers were produced by seeding a cell suspension comprising a mixture of untransformed C3H10T½ cells with the focus / tumour cells of interest. Culture dishes were examined for the presence of (+), (X/+), (X) and (-) foci.

frequencies of all other reconstructed focus categories; (+), (X) and (-). Examination of the focus frequencies produced by cells derived from the various categories of X-ray induced foci revealed that as for the confluent monolayers the (+) foci produced the highest frequency of all reconstructed foci (irrespective of focus category) and the highest frequency of reconstructed (+) and (-) foci, while the highest frequency of reconstructed (X) foci was produced by the (X) foci examined. The highest frequency of reconstructed (X/+) foci was shared by the (+) and (X/+) foci. Examination of the focus frequencies of the tumour cells from the various categories of X-ray induced foci revealed that the (+) tumour cells produced the highest frequency of all reconstructed foci (irrespective of focus category) and of reconstructed (+) foci, the highest frequency of reconstructed (X/+) foci was shared by both (+) and (X) tumour cells, highest frequency of reconstructed (X) was produced by the (X) tumour cells while the frequency of reconstructed (-) foci was similar for (+), (X/+) and (-) tumour cells.

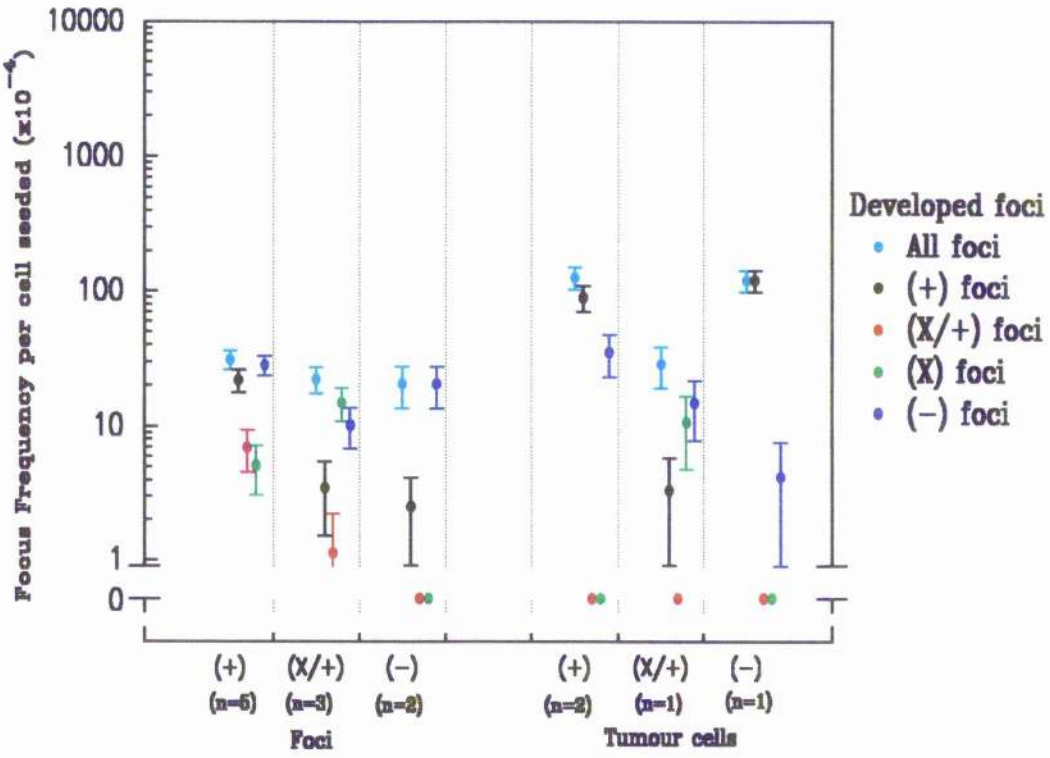
Figure 5.3.8 (a) illustrates the frequency of reconstructed foci induced by alpha - particle induced foci and their corresponding tumour cells on confluent monolayers of C3H10T½ cells. The (+) foci produced a lower frequency of reconstructed (+) foci and higher frequency of reconstructed (-) foci than the corresponding tumour cells. The only difference in the focus frequencies of the (X/+) foci and their corresponding tumour cells was a higher frequency of reconstructed (X) foci produced by the foci. The (-) foci had a lower frequency of reconstructed (+) foci than the corresponding tumour cells. Examination of the various categories of alpha - particle induced foci illustrated that the highest frequency of all reconstructed foci (irrespective of focus category), and of reconstructed (+), (X/+) and (-) foci was produced by the (+) foci. (X/+) foci produced the highest frequency of reconstructed (X) foci. Examination of the tumour cells showed that as for the foci the (+) tumour cells produced the highest frequency of all reconstructed foci (irrespective of focus category) and reconstructed (+) foci. There were no significant differences in the frequencies of the other foci produced.

Figure 5.3.8 (b) illustrates the frequency of reconstructed foci induced by alpha - particle induced foci and their corresponding tumour cells on mixed monolayers of C3H10T½ cells. The (+) focus cells produced a lower frequency of reconstructed (+)



Alpha-particle induced foci and tumour cells
Confluent monolayers

Figure 5.3.8 (a). Frequency of reconstructed foci produced by cells isolated from the alpha - particle induced foci and corresponding tumours when the focus / tumour cells were seeded on to confluent monolayers of C3H10T½ cells (n = number of cell lines examined in each category). Culture dishes were examined for the presence of (+), (X/+), (X) and (-) foci.



Alpha-particle induced foci and tumour cells
Mixed monolayers

Figure 5.3.8 (b). Frequency of reconstructed foci produced by cells isolated from the alpha - particle induced foci and corresponding tumours when the focus / tumour cells were seeded in monolayers of C3H10T½ cells (n = number of cell lines examined in each category). The mixed monolayers were produced by seeding a cell suspension comprising a mixture of untransformed C3H10T½ cells with the focus / tumour cells of interest. Culture dishes were examined for the presence of (+), (X/+), (X) and (-) foci.

foci and higher frequencies of reconstructed (X/+) and (X) foci than the corresponding tumour cells. There were no differences between the focus frequencies produced by the (X/+) foci and corresponding tumour cells. The (-) foci produced a lower frequency of reconstructed (+) foci and a higher frequency of reconstructed (-) foci than the corresponding tumour cells. Examination of the various categories of alpha - particle induced foci illustrated that the (+) foci had the highest frequency of reconstructed (+) and reconstructed (X/+) foci, whereas the (X/+) foci produced the highest frequency of reconstructed (X) foci, with no significant differences between the focus categories for the frequencies of reconstructed (-) foci. Examination of the tumour cells showed that the (+) and (-) tumour cells produced similar frequencies of all reconstructed focus categories (irrespective of focus category) and of reconstructed (+) foci. The highest frequency of reconstructed (-) and reconstructed (X) foci was observed for the (+) tumour cells and the (X/+) tumour cells respectively.

Data on number of reconstructed foci produced

The calculation of the focus frequency requires an estimation of the mean number of reconstructed foci per culture flask. It has been mentioned already in chapter three, with reference to the calculation of transformation frequency, that this could be done by counting all the foci produced but an overestimation of the number of reconstructed foci is likely due to the production of secondary foci by transformed cells which break away from the parent focus. Thus for the calculation of the focus frequency the number of culture flasks with or without foci is considered as explained in the sample calculations shown earlier in this section. The following figures compare the actual numbers of reconstructed foci produced by the original foci and their tumour cells on the mixed and confluent monolayers of C3H10T½ cells.

Figure 5.3.9 (a) presents the actual number of reconstructed foci produced by the X-ray induced focus cells on confluent monolayers of C3H10T½ cells. (+) foci reconstructed mostly foci categorised as (+) foci, (X) foci reconstructed mostly (X) foci while the (X/+) and (-) foci reconstructed few foci of any category. Figure 5.3.9 (b) presents the actual number of reconstructed foci induced by the X-ray induced focus cells on mixed monolayers of C3H10T½ cells and the same pattern was

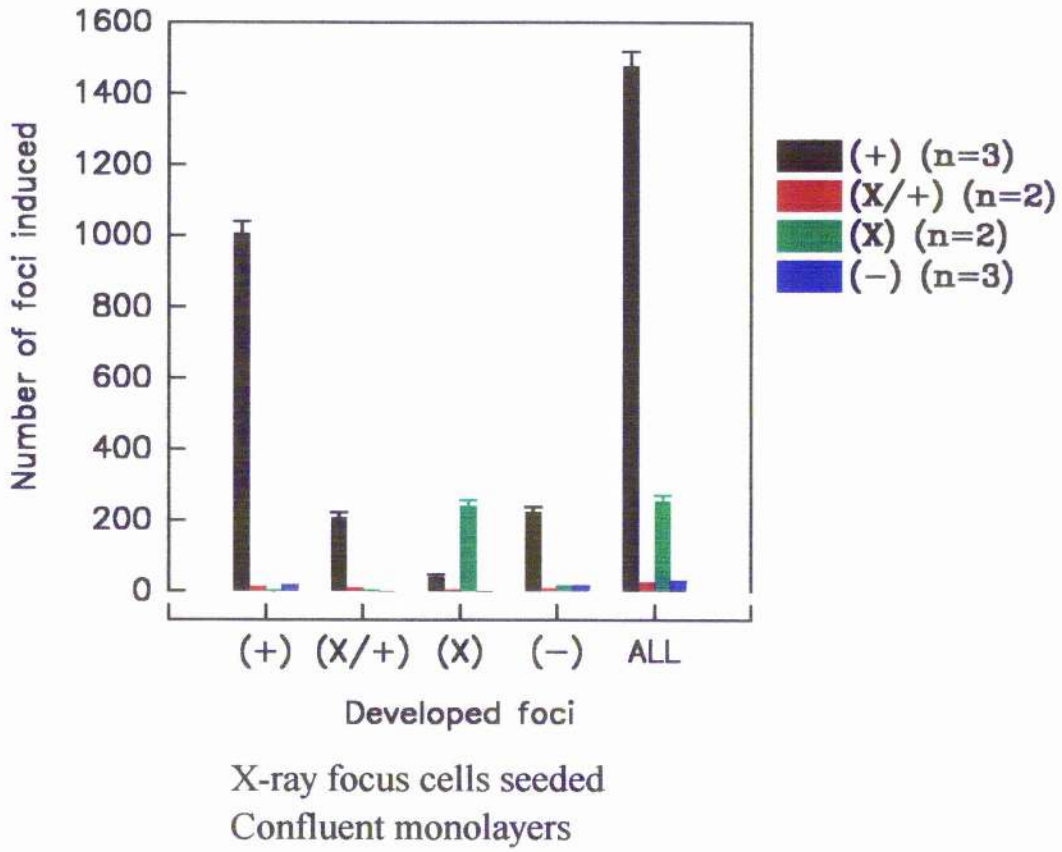


Figure 5.3.9 (a). Number of reconstructed foci induced on confluent monolayers of C3H10T½ cells seeded with X-ray induced focus cells (n = number of cell lines examined in each category). The data relate to the focus frequencies presented in figure 5.3.7a.

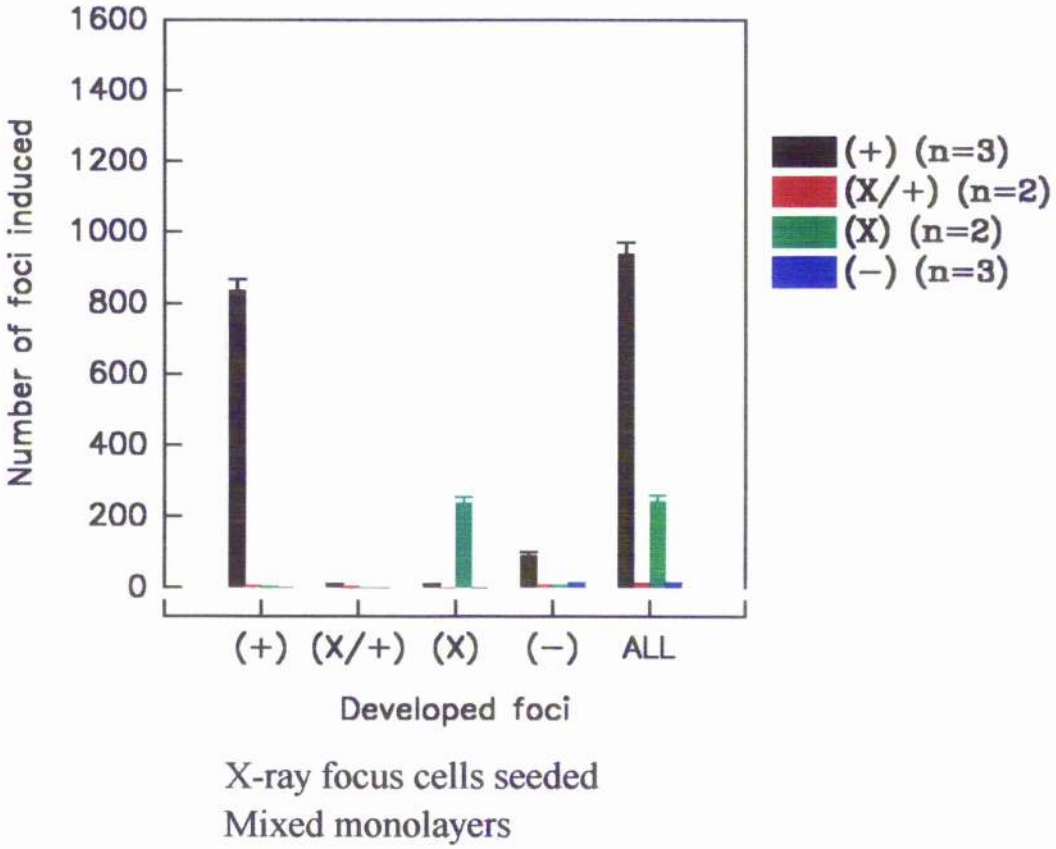


Figure 5.3.9 (b). Number of reconstructed foci induced on mixed monolayers (produced from a suspension of untransformed and focus C3H10T½ cells) of C3H10T½ cells seeded with X-ray induced focus cells (n = number of cell lines examined in each category). The data relate to the focus frequencies presented in figure 5.3.7b.

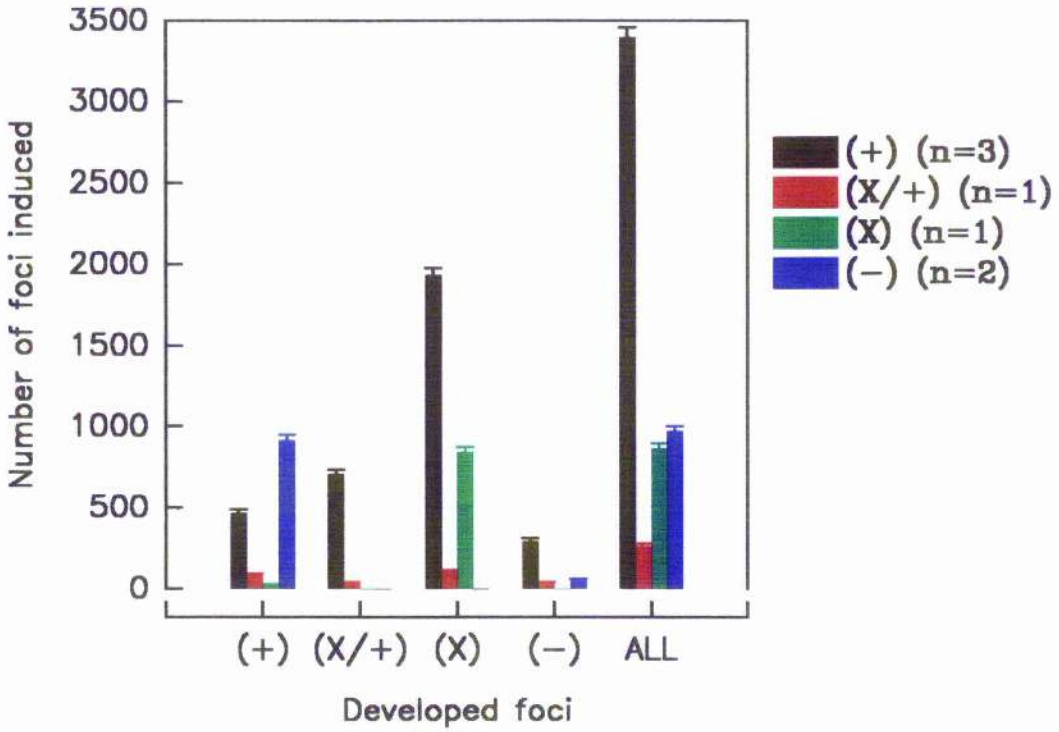
observed as for the confluent monolayers.

Comparison of the number of reconstructed foci produced on the mixed versus the confluent monolayers displayed that where foci were produced on the mixed monolayers a greater number was produced on the confluent monolayers with the exception of the number of reconstructed (X) foci produced by (X) foci where the number produced was similar for both types of monolayers. The total number of reconstructed foci for all the focus cells seeded was greater on the confluent monolayers.

Figure 5.3.10 (a) presents the actual number of reconstructed foci produced by the X-ray induced tumour cells on confluent monolayers of C3H10T½ cells. Most of the reconstructed foci produced by the (+) tumour cells were categorised as (X), followed by fewer numbers of reconstructed (X/+) and (+) foci and a small number of reconstructed (-) foci. (X/+) tumour cells produced low but similar numbers of all categories of reconstructed foci, whereas the (X) tumour cells produced a majority of reconstructed (X) foci and the (-) tumour cells produced a majority of reconstructed (+) foci.

Figure 5.3.10 (b) presents the actual number of reconstructed foci produced by the X-ray induced tumour cells on mixed monolayers of C3H10T½ cells. The (+) tumour cells produced equal numbers of reconstructed (X/+) and (X) foci with a smaller number of reconstructed (+) foci and few (-) foci. (X/+) tumour cells produced low but similar numbers of all categories of reconstructed foci, whereas the (X) tumour cells produced a majority of reconstructed (X) foci and the (-) tumour cells produced a majority of reconstructed (X) foci followed by lower numbers of reconstructed (+) and (-) foci.

Comparison of the number of reconstructed foci induced on the mixed versus confluent monolayers revealed that the (+) tumour cells produced more reconstructed (+) and (X/+) foci and less reconstructed (X) foci on the mixed monolayers, while the (X/+) foci produced low numbers of all categories on both mixed and confluent monolayers of C3H10T½ cells. No differences were noted for the (X) tumour cells, whereas the (-) tumour cells produced more reconstructed (+) and less reconstructed (X) foci on the confluent monolayers compared with the mixed monolayers. The total



X-ray tumour cells seeded
Confluent monolayers

Figure 5.3.10 (a). Number of reconstructed foci induced on confluent monolayers of C3H10T½ cells seeded with tumour cells derived from X-ray induced foci (n = number of cell lines examined in each category). The data relate to the focus frequencies presented in figure 5.3.7a.

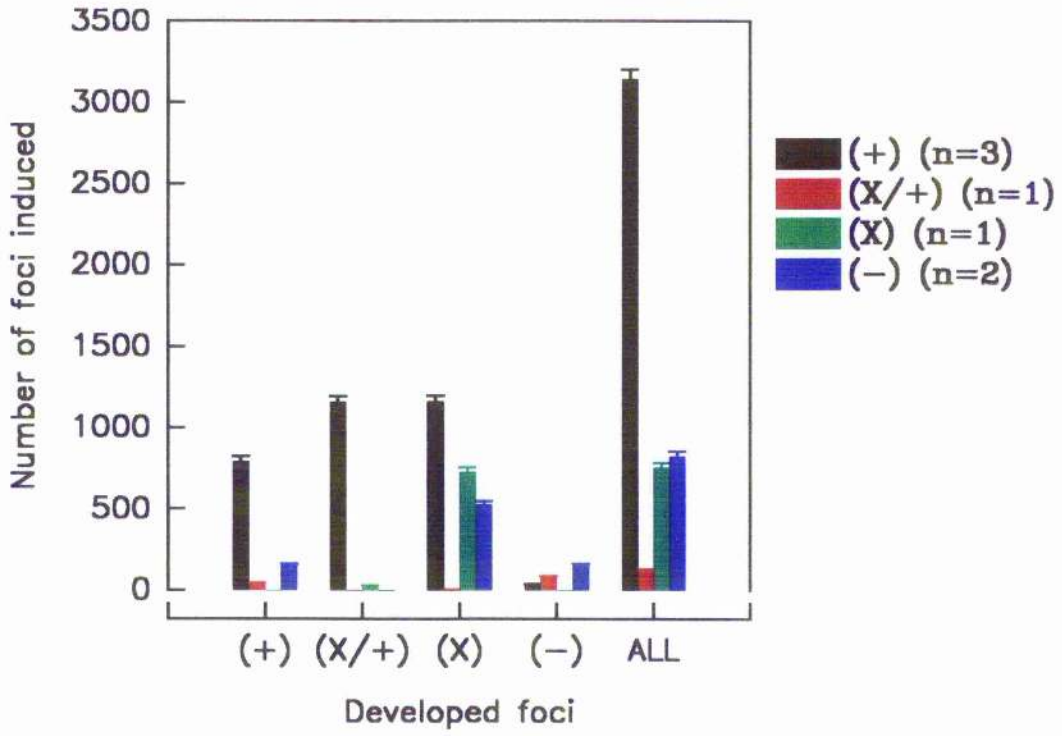


Figure 5.3.10 (b). Number of reconstructed foci induced on mixed monolayers (produced from a suspension of untransformed and tumour - derived C3H10T½ cells) of C3H10T½ cells seeded with tumour cells derived from X-ray induced foci (n = number of cell lines examined in each category). The data relate to the focus frequencies presented in figure 5.3.7b.

number of reconstructed foci for all the tumour cells seeded was similar for both mixed and confluent monolayers.

Comparison of figures 5.3.9 (a) and 5.3.10 (a), that is, the number of reconstructed foci produced by the X-ray induced focus cells versus the number induced by the tumour cells on confluent monolayers of C3H10T½ cells revealed that the (+) tumour cells produced less reconstructed (+) foci, more reconstructed (X/+) and (X) foci than the original focus cells, whereas the (X/+) tumour and focus cells showed the same pattern of producing few reconstructed foci of any category on the confluent monolayers. (X) tumour cells produced more reconstructed (X) foci, while the (-) tumour cells produced more reconstructed (+) foci than their corresponding focus cell lines. Examination of all the reconstructed foci produced by the cell lines without considering subsequent categorisation of these foci revealed that in all cases the tumour cells produced more foci than their corresponding focus cell lines.

Comparison of figures 5.3.9 (b) and 5.3.10 (b), that is, the number of reconstructed foci induced by the X-ray induced focus cells versus the number induced by the tumour cells on mixed monolayers of C3H10T½ cells revealed that the (+) tumour cells produced more reconstructed (X/+) and (X) foci, and fewer reconstructed (-) foci than the original focus cells with similar numbers of reconstructed (+) foci, whereas the (X/+) tumour and focus cells showed the same pattern seen in the confluent monolayers of producing few reconstructed foci of any category on the mixed monolayers. (X) tumour cells produced more reconstructed (X) foci, while the (-) tumour cells produced more reconstructed (X) and reconstructed (+) foci than their corresponding focus cell lines. Examination of all reconstructed foci produced by the cell lines without considering subsequent categorisation of these foci revealed that in all cases the tumour cells produced more foci than their corresponding focus cell lines.

Figure 5.3.11 (a) presents the actual number of reconstructed foci produced by the alpha - particle induced focus cells on confluent monolayers of C3H10T½ cells. The (+) foci produced equal numbers of reconstructed (+) and (-) foci followed by lower numbers of reconstructed (X/+) and (X) foci, while the (X/+) foci produced a majority of reconstructed (X) foci with a small number of (-) foci. No (X) foci were tested and the (-) foci produced few foci of any category.

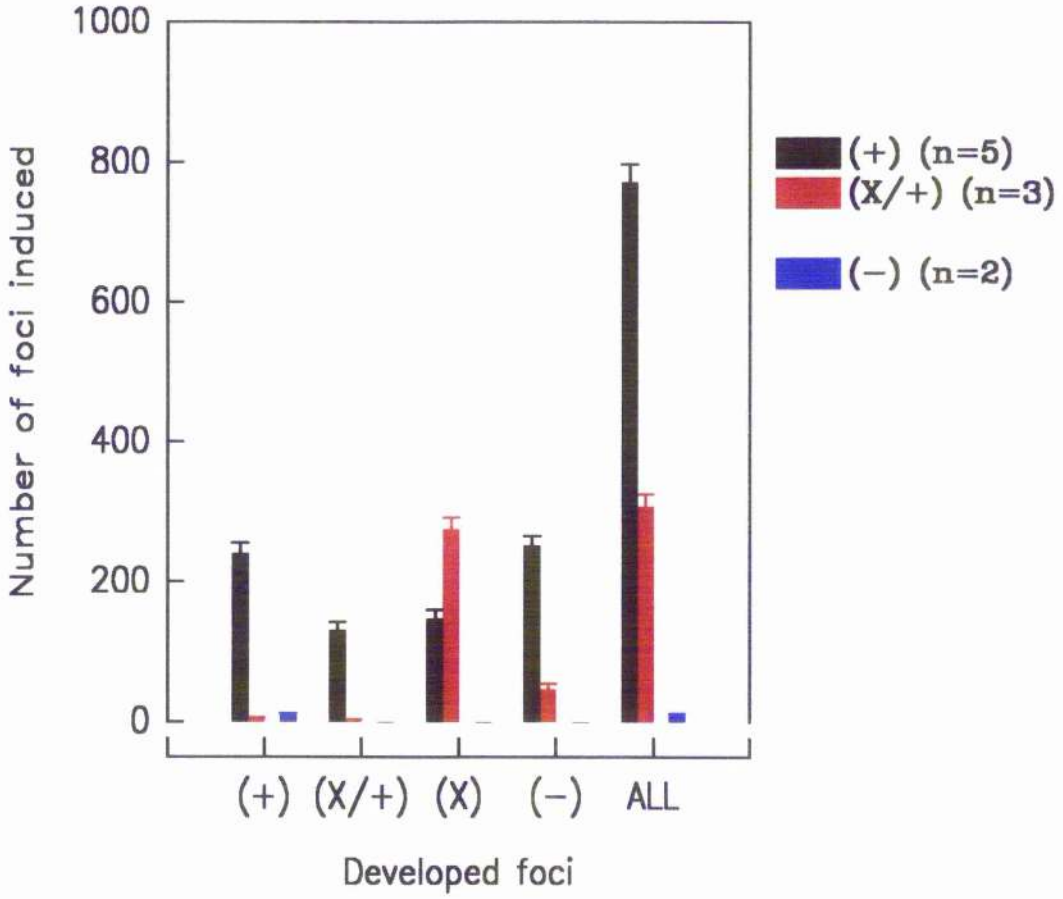


Figure 5.3.11(a). Number of reconstructed foci induced on confluent monolayers of C3H10T½ cells seeded with alpha - particle induced focus cells (n = number of cell lines examined in each category). These data relate to the focus frequencies presented in figure 5.3.8a.

Figure 5.3.11 (b) presents the actual number of reconstructed foci produced by the alpha - particle induced focus cells on mixed monolayers of C3H10T½ cells. The (+) foci reconstructed mostly (-) foci on the mixed monolayers followed by a smaller number of reconstructed (X/+) foci and also some reconstructed (+) foci, while the vast majority of the reconstructed foci produced by the (X/+) focus cells were categorised as (X). A small number of reconstructed foci mostly categorised as (-) were produced by the (-) focus cells.

Comparison of the number of foci induced on the mixed versus confluent monolayers revealed that the (+) focus cells produced more reconstructed (+) and (X) foci, less reconstructed (X/+) foci and approximately equal numbers of (-) foci on the confluent versus mixed monolayers. (X/+) foci produced a much greater number of reconstructed (X) foci and the (-) foci produced more reconstructed (-) foci on the mixed monolayers. The total number of reconstructed foci produced by the (+) foci was greater on the confluent monolayers, whereas the (X/+) and (-) foci produced more reconstructed foci in the mixed monolayers.

Figure 5.3.12 (a) presents the actual number of reconstructed foci produced by the alpha - particle induced tumour cells on confluent monolayers of C3H10T½ cells. The reconstructed foci produced by the (+) tumour cells were nearly all categorised as (+), with few reconstructed foci produced by the (X/+) tumour cells and almost all reconstructed foci produced by the (-) tumour cells categorised as (+) foci.

Figure 5.3.12 (b) presents the actual number of reconstructed foci produced by the alpha - particle induced tumour cells on mixed monolayers of C3H10T½ cells. In this case, the (+) and (-) tumour cells produced the same pattern as on the confluent monolayers while the (X/+) tumour cells produced more reconstructed foci, most of which were deemed to be (-) foci.

Comparison of the mixed and confluent monolayers showed the same pattern of reconstructed foci produced and except for the difference in the (X/+) tumour cells mentioned above, a much larger number of reconstructed foci were produced on the confluent monolayers than on the mixed monolayers.

Comparison of figures 5.3.11 (a) and 5.3.12 (a), that is, the number of reconstructed foci produced by the alpha - particle induced focus cells versus the

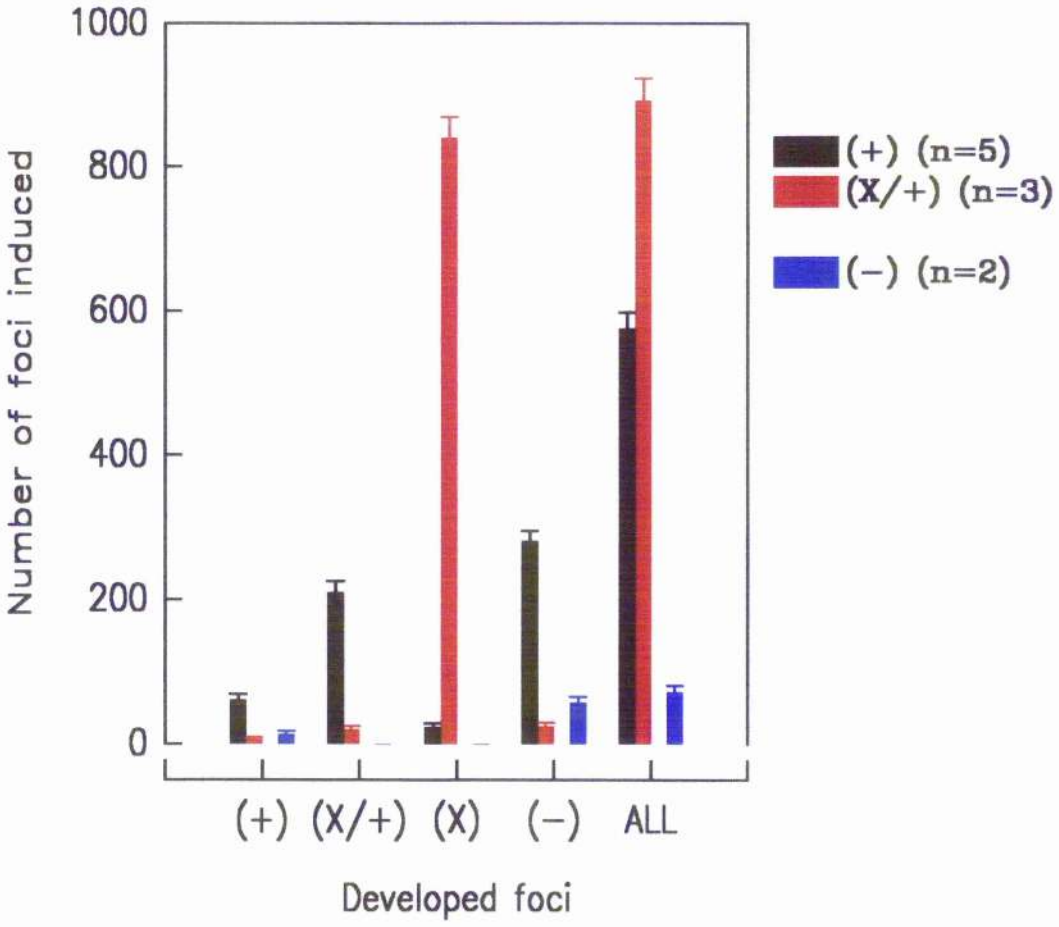


Figure 5.3.11 (b). Number of reconstructed foci induced on mixed monolayers produced from a suspension of untransformed C3H10T½ cells and alpha - particle induced C3H10T½ focus cells (n = number of cell lines examined in each category). These data relate to the focus frequencies presented in figure 5.3.8b.

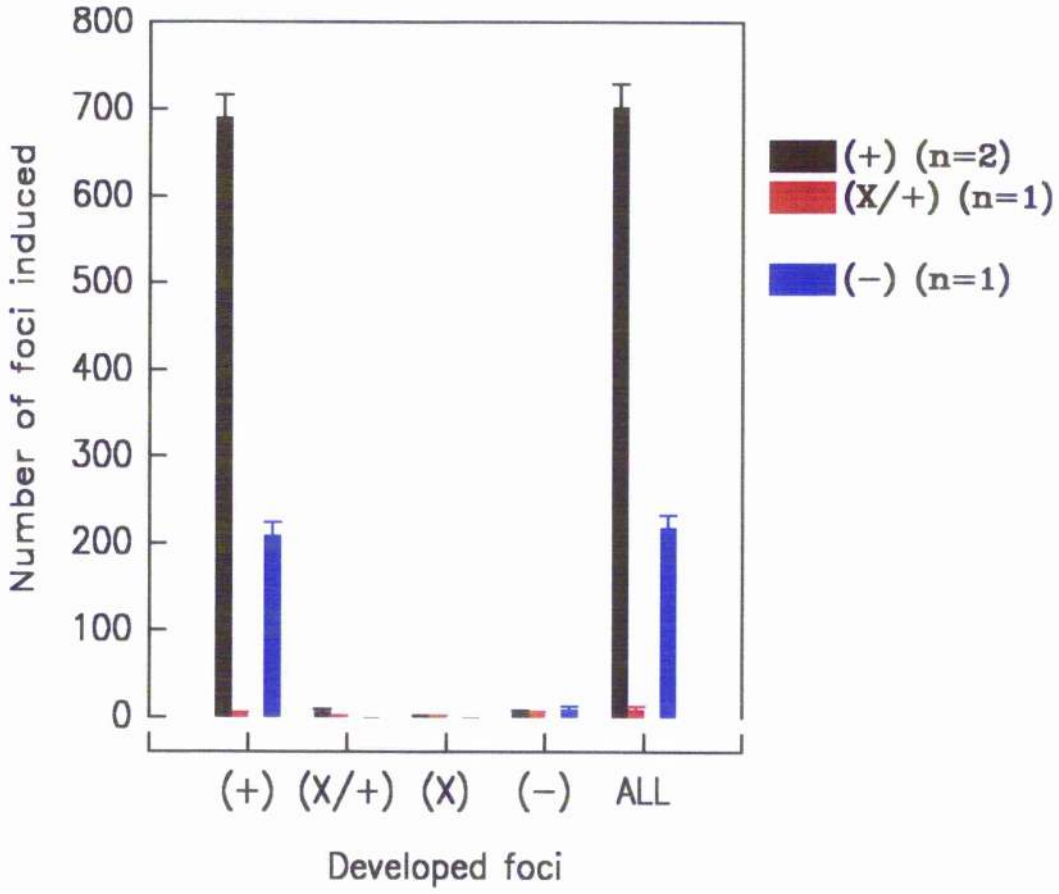


Figure 5.3.12 (a). Number of reconstructed foci induced on confluent monolayers of C3H10T½ cells seeded with tumour cells derived from alpha - particle induced foci (n = number of cell lines examined in each category). These data relate to the focus frequencies presented in figure 5.3.8a.

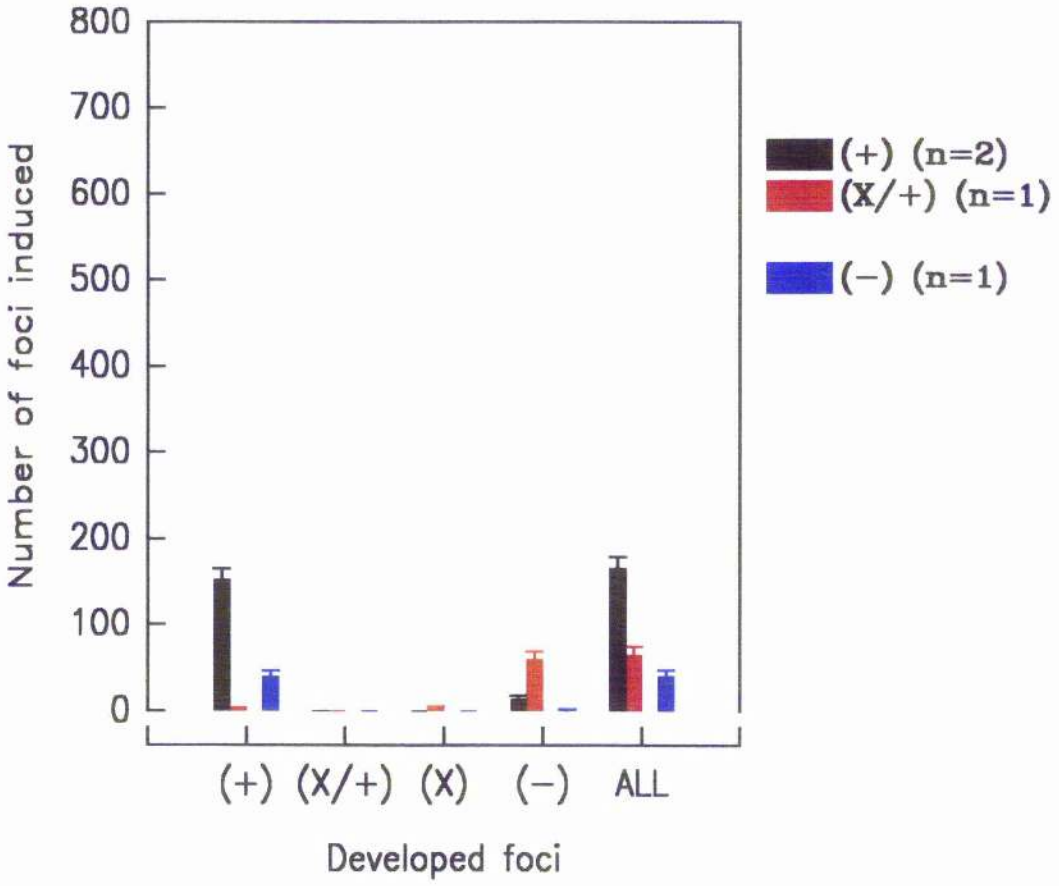
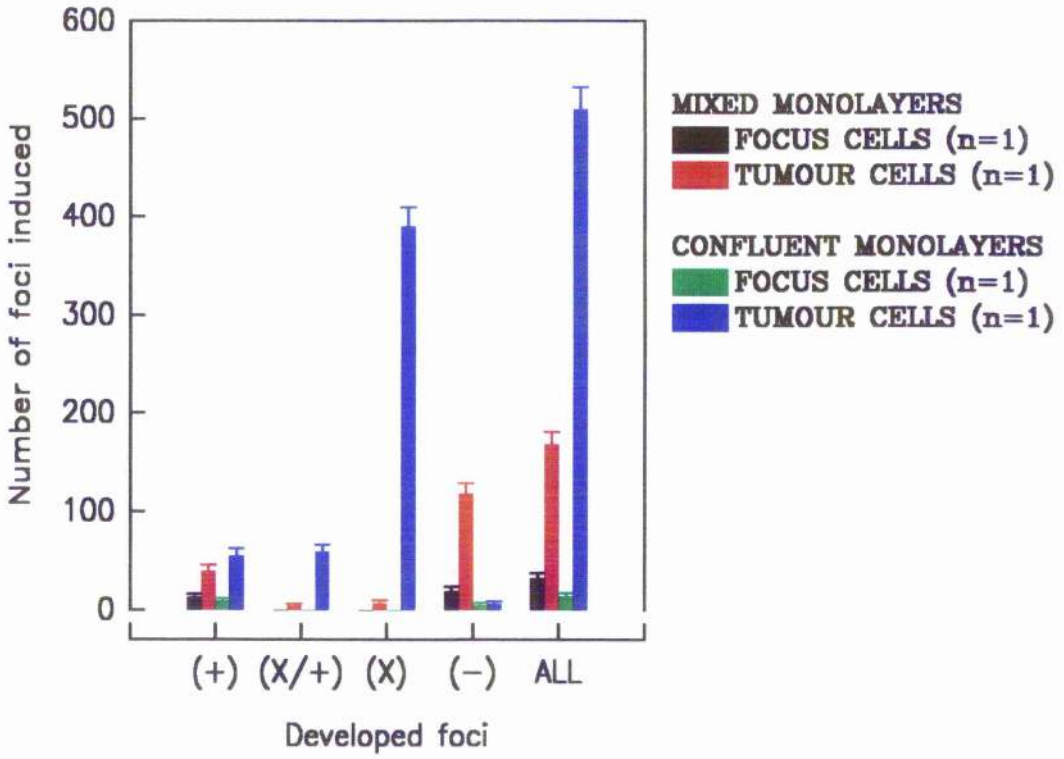


Figure 5.3.12 (b). Number of reconstructed foci induced on mixed monolayers produced from a suspension of untransformed C3H10T½ cells and tumour cells derived from alpha - particle induced C3H10T½ foci (n = number of cell lines examined in each category). These data relate to the focus frequencies presented in figure 5.3.8b.

number produced by the tumour cells on confluent monolayers of C3H10T½ cells revealed that the (+) tumour cells produced more reconstructed (+) foci, less (X/+), (X) and (-) foci than the original focus cells, whereas the (X/+) tumour cells produced fewer reconstructed (X) and (-) than the focus cells. (-) tumour cells produced more reconstructed (+) foci than the corresponding focus cells. Examination of all the foci produced by the cell lines without considering subsequent categorisation of these foci revealed that the (+) tumour cells and foci produced similar numbers of reconstructed foci, whereas the (X/+) focus cells produced more than the tumour cells and the reverse was true for the (-) foci and its tumour cells.

Comparison of figures 5.3.11 (b) and 5.3.12 (b), that is, the number of reconstructed foci produced by the alpha - particle induced focus cells versus the number produced by the tumour cells on mixed monolayers of C3H10T½ cells revealed that the (+) tumour cells produced more reconstructed (+), less (X/+) and (-) foci, and slightly less reconstructed (X) foci than the focus cells, whereas the (X/+) tumour cells produced less reconstructed (X) and more reconstructed (-) foci than the corresponding focus cells. (-) tumour cells produced more reconstructed (+) and less reconstructed (-) foci than their corresponding focus cell lines. Examination of all the reconstructed foci produced by the cell lines without considering subsequent categorisation of these foci revealed that (+) and (X/+) foci produced more reconstructed foci, while the (-) foci produced similar numbers of reconstructed foci to their corresponding tumour cells.

Figure 5.3.13 presents the data on the number of reconstructed foci produced by the spontaneous focus and its corresponding tumour cell line on both confluent and mixed monolayers of C3H10T½ cells. The focus cells produced few reconstructed foci on either the confluent or mixed monolayers, with approximately half of those produced categorised as (+) and half as (-) foci. However the tumour cells produced more reconstructed foci, most of which were categorised as (-) on the mixed monolayers and as (X) on the confluent monolayers. The total number of reconstructed foci produced was greatest for the tumour cells on the confluent monolayers followed by the tumour cells on the mixed monolayers.



Spontaneous focus and tumour cells

Figure 5.3.13. Number of reconstructed foci induced on confluent monolayers of C3H10T½ and on mixed monolayers (produced from a suspension of untransformed C3H10T½ and focus / tumour - derived cells) by the spontaneous focus and its corresponding tumour cell line (n = number of cell lines examined in each category). These data relate to the focus frequencies presented in figure 5.3.3b.

Discussion

The data for discussion can be divided into four areas, (a) comparison of the data on spontaneous versus X-ray versus alpha - particle induction of the original foci from which the C3H10T½ focus or tumour cell lines were derived, (b) comparison of the induction of foci on the mixed versus confluent monolayers of untransformed C3H10T½, (c) comparison of the data for the different categories of foci or tumour cells, that is, (+) versus (X/+) versus (X) versus (-), and lastly (d) the comparison of the focus cell lines with their corresponding tumour cells.

Spontaneous versus X-ray versus alpha - particle data

The first part of this discussion concentrates on the comparison of the X-ray, alpha -particle and spontaneous induction of the foci from which the focus and tumour cell lines were developed. In general, where differences occurred between radiation types the alpha - particle induced (X/+) and (-) foci produced higher focus frequencies for all the categories of reconstructed foci than the X-ray induced foci on both the mixed and confluent monolayers. The exception to this general trend was the production of (-) reconstructed foci by (-) foci on confluent monolayers where the X-ray induced foci produced a higher frequency of reconstructed foci. In general where differences occurred in the focus frequencies produced by (+) foci the X-ray induced foci produced higher frequencies of reconstructed foci than the corresponding alpha - particle induced foci. The exception to this was a higher frequency of reconstructed (-) foci produced by the alpha - particle induced (+) foci on the confluent monolayers. No alpha -particle induced (X) foci were examined and a spontaneous focus was available only in the (+) focus category.

Differences between the X-rays and alpha - particles were more apparent in the frequencies of reconstructed foci produced by the tumour cells. Tumour cells from the X-ray induced foci produced higher frequencies of reconstructed foci in almost all cases than the corresponding tumour cells from the alpha - particle induced foci. The only exceptions were evident on the mixed monolayers where the frequency of reconstructed (-) foci produced by tumour cells from the alpha -particle induced (X/+) foci and the frequency of reconstructed (+) foci produced by tumour cells from the

alpha -particle induced (-) foci were higher than those produced by the X-ray equivalent.

Comparison of the total number of foci actually produced by the different categories of X-ray or alpha - particle induced foci revealed on the confluent monolayers that the X-ray induced (+) foci produced more reconstructed (+) and (X/+) foci but less reconstructed (X) foci than the corresponding alpha - particle or spontaneous foci, while the X-ray induced (+) tumour cells produced more reconstructed (X/+) and (X) but less reconstructed (+) than the alpha - particle or spontaneous tumour cells. Differences between this pattern and that on the mixed monolayers was that the X-ray induced (+) foci produced fewer reconstructed (X/+) and (-) foci while still producing more reconstructed (+) foci than the equivalent alpha - particle induced foci, whereas the tumour cells produced more of all reconstructed foci except reconstructed (-) foci. The tumour cells developed from the spontaneous (+) focus produced the greatest number of reconstructed (-) foci on the mixed monolayers. Comparison of the total number of reconstructed foci induced by the (X/+) foci showed that the alpha - particle induced foci produced more reconstructed (X) and (-) foci on the confluent monolayers and more of all reconstructed foci on the mixed monolayers than the X-ray equivalent. No great differences were apparent in the number of reconstructed foci produced by the different radiation - induced (X/+) tumour cells on the mixed monolayers while the X-rays produced more reconstructed foci of all categories on the confluent monolayers. The (-) foci examined showed little difference between X-rays and alpha - particles in the number of reconstructed foci of each category; (+), (X/+), (X), (-) developed, while of the corresponding tumour cells the X-ray induced cells produced more reconstructed (+) foci on the confluent monolayers and more reconstructed (X) foci on the mixed monolayers yet similar numbers of the other reconstructed foci to that of the alpha - particle induced tumour cells.

The implication from the higher frequencies of foci produced by the alpha - particle induced foci compared to the X-ray induced or spontaneous foci is that these foci were less sensitive to the suppression effect of the untransformed C3H10T½ cells on both the mixed and confluent monolayers. Since one of the elements of

transformation is the loss of normal growth controls in transformed cells, allowing the cells to overcome some of the limitations to normal cell growth and thus making the transformed cells less reliant on their surroundings than normal cells, the findings described here suggest the alpha - particle induced foci may be 'more' transformed than their X-ray induced or spontaneous equivalent. The reverse pattern was observed for the tumour cells in that generally the tumour cells from the X-ray induced foci produced higher frequencies of the reconstructed foci than the tumour cells from the alpha - particle induced foci. Following the same logic as applied to the focus data the tumour cells from the X-ray induced foci are more 'transformed' than tumour cells from the alpha - particle induced foci. Since these cells are tumourigenic (the definitive test of transformation) one can surmise that if the ability to reconstruct foci is a transformation phenotype either it is not a constituent of all transformed or tumourigenic cells or that it is lost in tumourigenic cells resulting from alpha - particle induced foci. In the previous section of this chapter, tumourigenicity data were presented where the alpha - particle induced foci were less tumourigenic and took longer to produce tumours than the corresponding X-ray induced foci, and this was linked to possible genome instability induced by the alpha - particle irradiation (see discussion in section 5.2). This instability may still be present in the descendants of alpha - particle irradiated cells even after the cells have become tumourigenic and the genome instability may result in the loss of some characteristics of transformation, for example, the ability of the cells to reconstruct foci related to the original focus.

Interpretation of the data on the total number of foci produced by cells from each category of isolated foci needs careful examination as different numbers of foci or tumour cells were examined in different categories and there may be satellite foci included in the numbers due to the detachment of focus cells which relocated and produced new foci elsewhere on the culture dish. The differences in the numbers of reconstructed foci produced were more likely a function of the focus category than the radiation treatment. On the confluent monolayers, the X-ray induced foci and tumour cells developed the same number or in a few cases more reconstructed foci than the corresponding alpha - particle induced or spontaneous equivalent. One example was the (+) foci (sum product of three individual foci) which produced more reconstructed

(+) and (X/+) foci than the corresponding alpha - particle induced foci (sum product of five individual foci). This may be a case of the X-ray induced foci and tumour cells being more capable of producing satellite foci.

Induction of foci on mixed versus confluent monolayers of untransformed C3H10T½ cells

It has been reported in the literature that the expression of transformation of C3H10T½ cells can be suppressed by co-cultivating transformed cells with a large number of untransformed cells (Lloyd *et al.* 1978). This suppression effect was limited to a specific number of untransformed cells, above which transformed foci appeared again. In another publication where transformed cells were co-cultivated with untransformed cells (equivalent to the mixed monolayers referred to in this chapter) or seeded on to a monolayer of untransformed cells (equivalent to the confluent monolayers referred to in this chapter) a good correlation was found for the frequency of reconstructed foci produced by the two methods of cell seeding (Smith *et al.* 1993), a finding also supported by the data presented in this section. Data presented in this chapter allow for a comparison of the focus frequencies calculated for the various categories of foci and tumour cells induced by X-rays, alpha - particles or spontaneously on both the mixed and confluent monolayers. Comparison of the data from the mixed and confluent monolayers constitutes the next part of this discussion.

In general there were more similarities than differences in the focus frequencies calculated for the mixed versus confluent monolayers. Generally the X-ray induced foci; (+), (X/+), (X), (-), produced similar focus frequencies on both the mixed and confluent monolayers with the exception of increased frequencies of reconstructed (X/+) and (+) foci on the confluent monolayers by the (+) and (-) foci respectively. Variations in the resulting focus frequencies occurred for the tumour cells derived from the X-ray induced foci between mixed and confluent monolayers with some increased frequencies and some decreased frequencies of reconstructed foci observed for all categories of tumour cells. Less variation between mixed and confluent monolayers was observed for the alpha - particle induced foci and tumour cells. Of the alpha - particle induced foci only the (-) foci showed a difference between the mixed

and confluent monolayers of a lower frequency of reconstructed (-) foci on the confluent monolayers. The corresponding tumour cells showed differences between types of monolayer for the (+) cells only which showed a lower frequency of reconstructed (-) foci and higher frequencies of reconstructed (X/+) and (X) foci on the confluent monolayers compared to the mixed monolayers.

A further comparison of the mixed and confluent monolayers was done by comparison of the actual number of foci produced on each monolayer type by the different categories of foci examined. In cases where the X-ray induced foci developed foci on the mixed monolayers, more reconstructed foci were produced on the confluent monolayers with the sole exception of the number of reconstructed (X) foci induced by (X) foci, where the numbers produced were similar on both the mixed and confluent monolayers. The number of reconstructed foci produced by the corresponding tumour cells was less consistent as the tumour cells derived from (+) foci produced more reconstructed (+) and (X/+) foci and less reconstructed (X) foci on the mixed monolayers, whereas the tumour cells derived from the (-) foci produced less reconstructed (+) and more reconstructed (X) on the mixed monolayers. Only the tumour cells produced from the (X) foci developed similar numbers of foci on both mixed and confluent monolayers while tumour cells from the (X/+) foci produced few reconstructed foci on either monolayer type. The total number of reconstructed foci (all categories) produced by each category of tumour cells was similar for both mixed and confluent monolayers.

Comparison of the number of reconstructed foci produced by the alpha - particle induced foci and their corresponding tumour cells showed a more consistent pattern between the foci and tumour cells. The (+) foci created more reconstructed (+) and (X) foci with less reconstructed (X/+) foci on the confluent monolayers, while the (-) focus produced less reconstructed (-) foci on the confluent monolayers and both groups of tumour cells produced more reconstructed (+) foci only on the confluent monolayers. (X/+) foci produced a smaller number of reconstructed (X) foci on the confluent monolayers whereas the corresponding tumour cells produced few reconstructed foci of any category on the confluent monolayer and the majority of the reconstructed foci created on the mixed monolayer were categorised as (-) foci. The

(+) focus and tumour cells produced more reconstructed foci (sum of all categories) on the confluent monolayers while the (X/+) focus and tumour cells and the (-) focus cells all produced more reconstructed foci on the mixed monolayers. Tumour cells developed from the (-) foci created more foci on the confluent monolayers than on the mixed monolayers.

The similarity of the frequencies of reconstructed foci produced on the mixed versus confluent monolayers agrees with the results reported by Smith *et al.* (1993). Of the few exceptions in the data presented here half are increased frequencies on the confluent monolayers and half are increases in frequencies on the mixed monolayers. Examination of the total number of reconstructed foci produced revealed that the X-ray induced foci which produced foci on the mixed monolayers produced more reconstructed foci on the confluent monolayers. One possible explanation was the lack of competition for growth space when cells were seeded on to already confluent monolayers of cells compared to the competition for growth space when a mixed suspension of cells were seeded. Another possibility is a looser attachment of the focus cells to the monolayer of confluent cells allowing cells to become detached and relocated to produce satellite foci elsewhere in the culture. Of the tumour cells developed from the X-ray induced foci, the (+) and (-) cells showed differences in the number of reconstructed foci on the mixed versus confluent monolayers, an even distribution of some increases and some decreases in the number of foci of different categories. The (+) tumour cells showed an increase of reconstructed (+) foci and a decrease of reconstructed (X) foci on the mixed monolayers while the (-) tumour cells showed the reverse. Transformed cells seeded in a mixed suspension with a large number of untransformed cells had to compete for space on the culture dish far more than the transformed cells seeded directly on to an established confluent monolayer, where they 'simply' settle on the surface of the monolayer. One might expect that the transformed cells seeded on the confluent monolayers would find growth easier than those seeded in mixed suspensions by treating the monolayer as a feeder layer for cell attachment and nutrient supply. However seeding the cells in a mixed suspension was closer to the original transformation assay from which the original foci were first isolated, where a transformed cell was situated on the culture dish surrounded by

untransformed cells which provided a stimulus resulting in the continuous division of that transformed cell and the production of a focus. This preference for the production of foci on one type of monolayer over another appears to be a function of the focus category, and these categories are discussed in the next section.

Comparison of the different categories of foci and tumour cells

The next part of this discussion concentrates on comparing focus and tumour cells of (+) versus (X/+) versus (X) versus (-) foci. The section will first compare the X-ray induced foci, then the tumour cells, followed by the alpha - particle induced foci and then the tumour cells and lastly provides an overview of these data.

The first comparison is thus between the different categories of X-ray induced foci (+), (X/+), (X) and (-). The (+) foci were capable of reproducing foci of all other categories including its own on both the mixed and confluent monolayers, while the (X/+) and (-) foci produced few reconstructed foci of any category and the (X) foci reproduced only its own category of foci. Of these foci the highest frequency of reconstructed (+) and (X) foci were produced by the same category of cells seeded, that is the (+) foci reproduced mostly (+) foci and the (X) foci reproduced mostly (X) foci on both the mixed and confluent monolayers. The greatest number of reconstructed (+), (X/+) and (-) foci were produced by the (+) focus cells seeded while the largest number of reconstructed (X) foci was produced by the (X) focus cells seeded.

The next comparison in this part of the discussion is that of the frequency and number of reconstructed foci produced by the tumour cells derived from the X-ray induced foci. In this case, the (+) cells produced some of all categories of reconstructed foci on both the mixed and confluent monolayers while the (X/+) cells reproduced all categories on the confluent monolayers but no reconstructed (X/+) or (X) foci on the mixed monolayers. The (X) cells only reproduced (X) foci on the confluent monolayers and some reconstructed (X) and (X/+) foci on the mixed monolayers while the (-) cells reproduced all categories except reconstructed (X) and (X/+) foci on the confluent monolayers and reconstructed (X/+) on the mixed monolayers. The reconstructed foci most produced by the different categories of

tumour cells varied between mixed and confluent monolayers. Tumour cells from the (+) foci reproduced mostly (+) foci on the mixed monolayers and approximately equal frequencies of reconstructed (+) and (X) foci on the confluent monolayers, whereas the tumour cells from the (X/+) foci produced similar frequencies of reconstructed (+) and (-) foci on the mixed monolayers with no great differences apparent on the confluent monolayers. Tumour cells from the (X) foci produced mostly reconstructed (X) foci on both types of monolayers, while the tumour cells from the (-) foci produced mostly reconstructed (+) foci on the confluent monolayers with no major differences in the frequencies of reconstructed foci observed on the mixed monolayers. Comparison of the tumour cells to determine which produced the highest frequency of the four focus categories revealed that the highest frequency of reconstructed (+) foci was produced by the (+) cells on the mixed monolayers and shared by the (+) and (-) cells on the confluent monolayers. The greatest frequency of reconstructed (X/+) foci was produced by the (+) cells on the confluent monolayers and shared by the (+) and (X) cells on the mixed monolayers, while the highest frequency of reconstructed (X) foci was shared by the (+) and (X) cells on the confluent monolayers and produced by the (X) cells on the mixed monolayers. There were no great differences in the frequencies of reconstructed (-) foci produced by the tumour cells on the mixed monolayers while the highest frequency on the confluent monolayers was shared by the (+) and (X/+) cells.

Examination of the total number of foci produced by the different categories of tumour cells revealed that the (+) cells produced mostly (X) foci on the confluent monolayers and approximately equal numbers of reconstructed (X/+) and (X) foci on the mixed monolayers, while the (X/+) cells showed no great differences between numbers of reconstructed foci on either the mixed or confluent monolayers. Tumour cells from the (X) foci reproduced mostly (X) foci on both types of monolayers while the (-) cells produced mostly reconstructed (+) foci on the confluent monolayers and reconstructed (X) foci on the mixed monolayers. The largest number of reconstructed (+) foci was developed by the (-) cells on the confluent monolayers and by the (+) cells on the mixed monolayers. Tumour cells from the (+) foci produced the greatest number of both reconstructed (X) and (X/+) foci on both types of monolayers, while

the largest number of reconstructed (-) foci was produced by the (+) cells on the confluent monolayers and by the (-) cells on the mixed monolayers.

The next comparison in this section of the discussion is on the alpha-particle induced foci. There were no (X) foci so the comparison is between the (+), (X/+) and (-) foci. The (+) and (X/+) foci were able to reproduce some of all focus categories on both the mixed and confluent monolayers while the (-) foci produced frequencies of reconstructed (+) foci only on the confluent monolayers and of reconstructed (+) and (-) foci on the mixed monolayers. The (+) foci produced approximately equal frequencies of reconstructed (+) and (-) foci, while the (X/+) foci produced approximately equal frequencies of reconstructed (X) and (-) foci on both types of monolayer. The (-) foci produced no great differences in the frequencies of reconstructed foci on the confluent monolayers while the highest frequency of reconstructed foci produced on the mixed monolayers was of reconstructed (-) foci. On both the mixed and confluent monolayers the highest frequencies of reconstructed (+) and (X/+) foci were produced by the (+) foci with the highest frequency of reconstructed (X) foci produced by the (X/+) foci. The greatest frequency of reconstructed (-) foci was produced by the (+) foci on the confluent monolayers and shared by the (+) and (-) foci on the mixed monolayers.

Examination of the total number of reconstructed foci produced by each category of foci revealed that (+) foci produced approximately equal numbers of reconstructed (+) and (-) foci on the confluent monolayers and mostly reconstructed (-) on the mixed monolayers, while the (X/+) foci produced mostly reconstructed (X) foci on both types of monolayers. No great differences in the number of different reconstructed foci produced by the (-) foci were observed on the confluent monolayers where few foci of any category were produced. However a small number of mostly reconstructed (-) foci were evident on the mixed monolayers. The highest number of reconstructed (+), (X/+) and (-) foci were produced by the (+) foci seeded on both types of monolayer whereas the majority of the reconstructed (X) foci were produced by the (X/+) foci.

Examination of the frequency of reconstructed foci produced by the tumour cells from the alpha-particle induced foci revealed that the (+) cells only produced

some reconstructed (+) and (-) foci on the mixed monolayers and some of all categories of reconstructed foci on the confluent monolayers, while the (X/+) cells produced some of all categories on the confluent and some reconstructed (+), (X) and (-) foci on the mixed monolayers. Tumour cells from the (-) foci also produced some reconstructed (+) and (-) foci on both types of monolayers. The highest frequency of reconstructed foci produced by both the (+) and (-) cells was of reconstructed (+) foci on both the mixed and confluent monolayers with no great differences in the frequencies of reconstructed foci produced by the (X/+) cells.

Comparison of the total number of reconstructed foci produced by the tumour cells presented that the (+) and (-) cells reproduced mostly (+) foci on both types of monolayers while the (X/+) cells showed no great differences in the number of different foci produced on the confluent monolayers, but produced mostly reconstructed (-) foci on the mixed monolayers. The largest number of reconstructed (+) and (-) foci were produced by the tumour cells from the (+) and (X/+) foci respectively with no major differences in the number of reconstructed (X/+) or (X) foci produced.

Prior to the discussion of these data it seems appropriate to discuss the limitations of the calculation of the focus frequency as they influence data discussed here. The equation involves calculating the total number of viable cells using the knowledge of the number of cells seeded and the plating efficiency (details of the determination of plating efficiency were outlined in chapter two). Plating efficiencies were carried out in parallel with the focus reconstruction studies so that when the focus or tumour cells were seeded on the mixed or confluent monolayers, some cells were also seeded into culture flasks containing growth medium only, to determine the viability of the cells. The plating efficiencies of different cell lines varied considerably however a noticeable trend was a poor plating efficiency of some tumour cell lines compared to the original focus. This was most probably due to a poor affinity of the tumour cells for the plastic cell culture surface, an idea supported in some cases by the same tumour cells producing numerous foci on the mixed or confluent monolayers. Plating efficiency assays can only assess the viability of the cells which remain attached to the culture surface and may underestimate the viability of the cells when

seeded in the actual focus reconstruction studies. This is a limitation of calculations of focus frequencies which can be restricted in some cases by combining as many different cell lines as possible in a particular category as has been done in previous sections of this discussion. However the influence is greater when examining individual foci and individual tumour cell lines and caution should be exercised in comparisons of individual cell lines.

A few threads which seem to link the categories of X-ray induced foci and tumour cells and the alpha-particle induced foci and tumour cells are that for the most part, (+) foci and tumour cells were more able to reproduce all categories of reconstructed foci while the (X) foci and tumour cells (X-ray data only) seemed able to produce reconstructed (X) or (-) foci only. Of the remaining two focus categories the (X/+) cells were sometimes able to produce foci of all categories, however where a preference was apparent, they favoured (+), (X) or (-) foci rather than (X/+) foci. The (-) foci and their tumour cells tended not to produce (X/+) or (X) foci, concentrating instead on the production of reconstructed (+) and (-) foci.

The first possibility to explain the ability of the (+) foci to reproduce all the other categories of reconstructed foci was that the foci consisted of a mixture of cells which were at different stages of transformation. This implies that (+), (X/+), (X) and (-) foci are different stages of the same process. It is generally agreed that transformation and carcinogenesis are multistage processes (for example, Hall 1994, Fearon and Vogelstein 1990), thus one could speculate that the (+) foci are closer to the end of the transformation line (complete transformation / tumourigenicity), while the (-) foci are nearer the beginning with the (X/+) and (X) foci somewhere in between. Since the (X/+) foci were capable of producing all the focus categories under some conditions whereas the (X) foci tended to produce (X) or (-) foci, one could speculate the (X) foci are closer to the (-) foci, near the beginning of the 'transformation line' while the (X/+) foci lie between the (X) and (+) foci on the same line.

If the different focus categories represent different stages of transformation the question must be asked if they all aspire to reach the end of the line and become fully transformed. So now one examines the data provided by the tumour cells. A selection

process has been undergone by the focus cells and this selection process tends to favour the isolation of fully transformed, that is, tumourigenic cells. If all the stages of transformation aspire to one phenotype and the (+), (X/+), (X) and (-) are different stages, then one would expect a predominance of one phenotype in the reconstruction studies. Yet again a similar pattern was observed for the different categories of tumour cells as seen for the foci. So the selection of tumourigenic cells has not defined only one phenotype. Revision of the above theory and one that fits with the data would be that the (+), (X/+) and (X) may be different ends of the line, that is, there are different pathways to transformation. This does not necessarily indicate independent pathways, it is more likely the pathways are interlinked. One of the most well characterised systems of the multistage process of carcinogenesis is that of colorectal cancer. This cancer is understood to evolve from the combination of a number of changes in the cell genome, with mutations in at least four to five genes required for the formation of a malignant tumour and fewer changes required for a benign tumour (Fearon and Vogelstein 1990). The important point to note for this discussion is that it is the accumulation of changes that is important and not necessarily the order of occurrence of those changes. If the same is true for transformation of the C3H10T½ cells then the different phenotypes observed may be the result of different combinations of changes or different orders of occurrence of those changes in the cell genome or environment. At present these necessary changes have not been characterised although a number of authors have examined oncogene and tumour suppressor genes and found no apparent link between these and transformation (Shuin *et al.* 1986, Borek *et al.* 1987, Krolewski *et al.* 1994, Privitera *et al.* 1990, Thomas *et al.* 1991). These authors favour the involvement of epigenetic processes and some as yet unidentified gene changes.

Comparison of foci and their corresponding tumour cells.

The next part of this discussion compares the foci with their corresponding tumour cells. Examination of the X-ray induced (+) foci presented higher frequencies of all categories of reconstructed foci produced by the tumour cells compared to the original foci on both the mixed and confluent monolayers, when the foci / tumour cells were examined as a group. When the three individual foci were compared with their

individual tumour cells it was revealed that where differences occurred the same pattern was evident where the tumour cells produced higher frequencies of a focus category than its focus cell line. The same difference was observed for the X-ray induced (X/+), (X) and (-) foci and their tumour cell lines. The only exception was one of the X-ray induced (-) focus cell lines produced a higher frequency of reconstructed (-) foci than its corresponding tumour cells.

In many ways the same pattern was found for the alpha-particle induced foci and their tumour cells. Examination of the groups (combined data of a number of foci) of (+), (X/+) and (-) foci and their corresponding tumour cell lines revealed some cases where both for the mixed and confluent monolayers the foci produced higher frequencies of reconstructed foci than the tumour cells, the opposite to that observed for the X-ray induced focus and tumour cells and other cases where the reverse occurred. Thus the differences between the foci and tumour cells were not as clear for the alpha-particles as they appeared to be for the X-rays. The only difference observed between the spontaneous (+) focus and its tumour cell line was the decreased frequency of reconstructed (+) foci produced by the foci on both the mixed and confluent monolayers.

The overall trend in the foci versus tumour cells comparison was that for the X-rays and alpha-particles the tumour cells produced higher frequencies of reconstructed foci than their corresponding focus cells while the reverse was true in a number of cases for the alpha-particle induced foci and tumour cells. Results obtained for the X-rays were expected as the foci were a more heterogeneous population of normal and transformed cells than the tumour cells which would have selected a predominance of tumourigenic, fully transformed cells. The same selection process was assumed to have occurred for the alpha-particle induced foci and tumour cells and seems to have happened in some, but not all cases. One possibility is that under the conditions of this assay some groups of tumour cells were less able to demonstrate their ability to reconstruct foci than the original focus cells and this could be explained by a possibly reduced capacity of the tumour cells to attach to the monolayers and thus produce a focus. Another possible explanation is that on the route to tumourigenicity the ability to produce the phenotypic changes manifested in the foci

was lost in some cases. It is worth noting that in the previous section in this chapter on tumourigenicity the alpha-particle induced foci were less tumourigenic and took longer to produce tumours than the corresponding X-ray induced foci, and this was linked to possible genome instability induced by the alpha-particle irradiation (see discussion in section 5.2). Perhaps this instability is still present even after the cells have become tumourigenic and the ability of the cells to change morphology is also an unstable parameter. This point will be discussed further in the final discussion at the end of chapter five on all the data presented on the foci and tumour cells of X-rays and alpha-particles.

Summary

There appeared to be a number of trends evident in the focus reconstruction studies. While cells isolated from the alpha-particle induced foci produced higher frequencies of reconstructed foci per viable cell (but lower total numbers of foci), in most cases, than the X-ray induced or spontaneous equivalent the tumour cells presented the opposite trend with higher frequencies of reconstructed foci produced by X-rays compared to alpha-particles. The frequencies of reconstructed foci on the mixed and confluent monolayers were similar for the most part, with a greater number of foci usually produced on the confluent monolayers. Examination of the different focus categories indicated that the (+) focus and tumour cells were capable of producing all other categories of foci and did so on almost all occasions. Of the other categories, the (X) cells tended to reproduce (X) or (-) foci, while the (X/+) cells tended to produce any category except its own, (X/+), and the (-) cells would only produce (+) or (-) foci. Comparison of the individual foci and their tumour cells showed that for X-rays and alpha-particles the foci generally produced lower frequencies of reconstructed foci than their corresponding tumour cells although the reverse was true in some cases for the alpha-particles.

Appendices

Focus frequencies ($x10^{-4}$) on Confluent Monolayers (± S.E.)								
Focus Type	Cell Line	Total cells	Viable cells	All Foci	(+) Foci	(X/+) Foci	(X) Foci	(-) Foci
(+)	X1	9024	2504	207.8 ± 28.8	0 ± 0	207.8 ± 28.8	29.1 ± 10.8	13.1 ± 7.2
	X9	14232	3618	2.8 ± 2.8	0 ± 0	0 ± 0	0 ± 0	2.8 ± 2.8
	X19	15816	3917	260.5 ± 25.8	260.5 ± 25.8	0 ± 0	0 ± 0	39.1 ± 10
	Combined	39072	10039	89.2 ± 9.4	39.8 ± 6.3	19.6 ± 4.4	6.3 ± 2.5	18.3 ± 4.3
	α10	8004	2631	113.3 ± 20.8	113.3 ± 20.8	8.3 ± 5.6	0 ± 0	81.7 ± 17.6
	α19	12120	2966	100.5 ± 18.4	72.5 ± 15.6	3.5 ± 3.4	0 ± 0	100.5 ± 18.4
	α1	4782	1890	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	α12	8502	2627	113.5 ± 20.8	50.2 ± 13.8	0 ± 0	8.3 ± 5.6	63.3 ± 15.5
	α18	7170	2160	138 ± 25.3	16 ± 8.6	48.6 ± 15	48.6 ± 15.0	4.8 ± 4.7
	Combined	40578	12274	69.9 ± 7.5	37.3 ± 5.5	8.8 ± 2.7	7.9 ± 2.5	35.7 ± 5.4
	STR	7914	2698	48.9 ± 13.5	24 ± 9.4	0 ± 0	0 ± 0	24 ± 9.4
(+) TCL	X1 TCL	6579	54	6212.8 ± 1074.9	0 ± 0	1499.4 ± 528.6	6212.8 ± 1076	0 ± 0
	X9 TCL	6530	1271	262.4 ± 45.5	63.3 ± 22.3	79.1 ± 25	63.3 ± 22.3	63.3 ± 22.3
	X19 TCL	6592	578	577 ± 99.9	577 ± 99.9	0 ± 0	18 ± 17.7	37.6 ± 25.5
	Combined	19701	1903	751.1 ± 62.8	137 ± 26.8	83.1 ± 21	147.4 ± 27.8	47 ± 15.7
	α10 TCL	9636	915	234.9 ± 50.7	114.8 ± 35.4	23.9 ± 16.2	11.4 ± 11.2	37.7 ± 20.3
	α19 TCL	9612	1442	206.8 ± 37.9	206.8 ± 37.9	0 ± 0	0 ± 0	0 ± 0
	Combined	19248	2357	253 ± 32.8	160 ± 26	8.9 ± 6.1	4.3 ± 4.3	13.6 ± 7.6

Focus frequencies ($x10^{-4}$) on Confluent Monolayers (± S.E.)								
Focus Type	Cell Line	Total cells	Viable cells	All Foci	(+) Foci	(X/+) Foci	(X) Foci	(-) Foci
	STR TCL	8374	4168	79.9 ± 13.9	19.3 ± 6.8	19.3 ± 6.8	80 ± 13.9	5.2 ± 3.5
(X/+)	X4	8988	4886	18.1 ± 6.1	9.26 ± 4.4	4.3 ± 3	4.3 ± 3	6.72 ± 3.71
	X12	4998	2230	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Combined	13986	7116	10.9 ± 3.9	6 ± 2.9	2.9 ± 2	2.9 ± 2	4.4 ± 2.5
	α5	8628	3458	86.2 ± 15.8	10 ± 5.4	6.3 ± 4.3	38.1 ± 10.5	86.2 ± 15.8
	α13	7524	2469	33.7 ± 11.7	0 ± 0	0 ± 0	33.7 ± 11.7	4.3 ± 4.1
	α22	7080	3606	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Combined	23232	9533	26.2 ± 5.2	3.3 ± 1.9	2.2 ± 1.5	18.6 ± 4.4	15.3 ± 4
(X/+) TCL	X4 TCL	9547	2387	139.7 ± 24.2	42.1 ± 13.3	26.4 ± 10.5	64.2 ± 16.4	42.1 ± 13.3
	α5 TCL	4788	3089	19 ± 7.8	10.6 ± 5.9	3.3 ± 3.3	3.3 ± 3.3	10.6 ± 5.9
(X)	X2	2970	2969	112.3 ± 19.5	3.5 ± 3.4	7.3 ± 5	112.3 ± 19.5	16.1 ± 7.4
	X11	3498	3500	9.4 ± 5.2	0 ± 0	0 ± 0	6.1 ± 4.2	0 ± 0
	Combined	6468	6469	34.8 ± 7.3	1.6 ± 1.6	3.2 ± 2.2	31.7 ± 7	6.6 ± 3.2
(X) TCL	X2 TCL	4995	2217	150.4 ± 26.0	36.3 ± 12.8	0 ± 0	150.4 ± 26	0 ± 0
(-)	X6	62250	25510	0.4 ± 0.4	0 ± 0	0 ± 0	0 ± 0	0.4 ± 0.4
	X14	9576	2107	16.4 ± 8.8	10.4 ± 7	0 ± 0	0 ± 0	10.4 ± 7.0
	X18	1860	149	559 ± 193.8	70.2 ± 68.7	0 ± 0	0 ± 0	559 ± 193.8
	Combined	73686	27766	4 ± 1.2	1.1 ± 0.6	0 ± 0	0 ± 0	3.5 ± 1.1

Focus frequencies ($x10^{-4}$) on Confluent Monolayers (± S.E.)								
Focus Type	Cell Line	Total cells	Viable cells	All Foci	(+) Foci	(X/+) Foci	(X) Foci	(-) Foci
	α9	8244	2598	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	α14	9648	1447	15.1 ± 10.2	15.1 ± 10.2	0 ± 0	0 ± 0	0 ± 0
	Combined	17892	4045	5.2 ± 3.6	5.2 ± 3.6	0 ± 0	0 ± 0	0 ± 0
(-) TCL	X14 TCL	9612	2115	141 ± 25.8	78.7 ± 19.3	0 ± 0	0 ± 0	16.3 ± 8.8
	X18 TCL	9636	771	386.8 ± 70.8	386.8 ± 70.8	0 ± 0	0 ± 0	0 ± 0
	Combined	19248	2886	264.3 ± 30.3	173 ± 24.4	0 ± 0	0 ± 0	11.1 ± 6.2
	α9 TCL	9564	2487	119.9 ± 22	119.9 ± 22	0 ± 0	0 ± 0	4.2 ± 4.1
Untransformed cells		33693	16728	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Appendix 5.3.1: Frequencies of foci ± standard error (S.E.) produced on the confluent monolayers. The values are given for individual cell lines of all foci produced (irrespective of category) as well as values for the frequencies of foci of each category produced on the confluent monolayers of C3H10T½ cells. STR is the abbreviation for the spontaneous cells, α denotes alpha-particle induced foci, X denotes X-ray induced foci, TCL is the abbreviation for tumour cell lines. Combined data are presented in figures 5.3.3 to 5.3.8.

Focus Frequencies ($x10^{-4}$) on Mixed Monolayers (± S.E.)								
Focus Type	Cell Line	Total cells	Viable cells	All Foci	(+) Foci	(X/+) Foci	(X) Foci	(-) Foci
(+)	X1	9024	2504	79 ± 17.8	0 ± 0	13.1 ± 7.2	13.1 ± 7.2	49.8 ± 14.1
	X9	14232	3618	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	X19	15816	3917	260.5 ± 25.8	207.4 ± 23.0	0 ± 0	0 ± 0	43.5 ± 10.6
	Combined	39072	10039	63.8 ± 8	36.3 ± 6	3.1 ± 1.7	3.1 ± 1.7	26.7 ± 5.2
	α10	8004	2631	81.7 ± 17.6	113.3 ± 20.8	0 ± 0	13.1 ± 7.1	81.7 ± 17.6
	α19	12120	2966	28 ± 9.7	21.8 ± 8.6	0 ± 0	0 ± 0	28.0 ± 9.7
	α1	4782	1890	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	α12	8502	2627	31.7 ± 11	24.6 ± 9.7	8.3 ± 5.6	13.1 ± 7.1	31.6 ± 11
	α18	7170	2160	38.5 ± 13.4	0 ± 0	38.5 ± 13.4	0 ± 0	29.9 ± 11.8
	Combined	40578	12274	31.1 ± 5	21.8 ± 4.2	7 ± 2.4	5.1 ± 2	28.3 ± 4.8
	STR	7914	2698	38.9 ± 12.0	12.8 ± 6.9	0 ± 0	0 ± 0	38.9 ± 12.0
(+) TCL	X1 TCL	6073	49	6135.6 ± 1123.6	1711.5 ± 593.4	1711.5 ± 593.4	6135.6 ± 1123.6	0 ± 0
	X9 TCL	6530	1271	262.4 ± 45.5	191.5 ± 38.8	0 ± 0	17.1 ± 11.6	49.7 ± 19.8
	X19 TCL	6085	533	559.8 ± 102.5	559.8 ± 102.5	0 ± 0	19.6 ± 19.2	64.8 ± 34.9
	Combined	18688	1853	721.4 ± 62.4	306 ± 40.6	35.4 ± 13.8	103.9 ± 23.7	48.7 ± 16.2
	α10 TCL	9636	915	70.7 ± 27.8	23.9 ± 16.2	0 ± 0	0 ± 0	53.2 ± 24.1
	α19 TCL	9612	1442	206.8 ± 37.9	206.8 ± 37.9	0 ± 0	0 ± 0	23.9 ± 12.9
	Combined	19248	2357	125.5 ± 23	89.1 ± 19.4	0 ± 0	0 ± 0	35.1 ± 12.2
	STR TCL	7730	3892	76.6 ± 14.0	27 ± 8.3	8.9 ± 4.8	12.5 ± 5.7	76.6 ± 14.0

Focus Frequencies ($x10^{-4}$) on Mixed Monolayers (± S.E.)								
Focus Type	Cell Line	Total cells	Viable cells	All Foci	(+) Foci	(X/+) Foci	(X) Foci	(-) Foci
(X/+)	X4	8988	4886	12 ± 5	4.34 ± 2.98	2.1 ± 2.1	0 ± 0	6.72 ± 3.707
	X12	5276	2301	4.5 ± 4.4	0 ± 0	0 ± 0	0 ± 0	4.46 ± 4.4
	Combined	14264	7187	9.1 ± 3.6	2.9 ± 2	1.4 ± 1.4	0 ± 0	5.9 ± 2.9
	α5	7909	3189	72.2 ± 15.0	11.2 ± 5.9	0 ± 0	28.7 ± 9.5	50.5 ± 12.6
	α13	6897	2230	29.9 ± 11.6	0 ± 0	4.7 ± 4.6	29.9 ± 11.6	0 ± 0
	α22	7080	3606	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Combined	21886	9025	22.2 ± 5	3.5 ± 2	1.1 ± 1.1	14.8 ± 4.1	10.2 ± 3.4
(X/+) TCL	X4 TCL	8813	2261	58.3 ± 16.1	58.3 ± 16.1	0 ± 0	4.6 ± 4.5	36.8 ± 12.8
	α5 TCL	4788	3089	28.7 ± 9.6	3.3 ± 3.3	0 ± 0	10.6 ± 5.9	14.6 ± 6.9
(X)	X2	2970	2969	112.3 ± 19.5	3.5 ± 3.4	0 ± 0	112.3 ± 19.5	0 ± 0
	X11	3498	3500	12.9 ± 6.1	0 ± 0	0 ± 0	9.4 ± 5.2	2.9 ± 2.9
	Combined	6468	6469	38.1 ± 7.7	1.6 ± 1.6	0 ± 0	34.8 ± 7.3	1.6 ± 1.6
(X) TCL	X2 TCL	4611	2007	148.6 ± 27.2	0 ± 0	52.4 ± 16.2	148.6 ± 27.2	0 ± 0
(-)	X6	62250	25510	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	X14	9576	2107	23.1 ± 10.5	0 ± 0	0 ± 0	0 ± 0	23.1 ± 10.5
	X18	1705	136	364.5 ± 163.5	0 ± 0	0 ± 0	0 ± 0	364.5 ± 163.5
	Combined	73531	27753	3.1 ± 1.1	0 ± 0	0 ± 0	0 ± 0	3.1 ± 1.1
	α9	8244	2598	32 ± 11.1	0 ± 0	0 ± 0	0 ± 0	32 ± 11.1

Focus Frequencies ($x10^{-4}$) on Mixed Monolayers (± S.E.)								
Focus Type	Cell Line	Total cells	Viable cells	All Foci	(+) Foci	(X/+) Foci	(X) Foci	(-) Foci
	α14	9648	1447	7.2 ± 7.1	7.2 ± 7.1	0 ± 0	0 ± 0	7.22 ± 7.1
	Combined	17892	4045	20.5 ± 7.1	2.5 ± 2.5	0 ± 0	0 ± 0	20.5 ± 7.1
(-) TCL	X14 TCL	9612	2115	141 ± 25.8	0 ± 0	0 ± 0	141 ± 25.8	0 ± 0
	X18 TCL	7227	578	342 ± 76.9	342 ± 76.9	0 ± 0	0 ± 0	342 ± 77
	Combined	16839	2693	237.4 ± 29.7	43.6 ± 12.7	0 ± 0	66.1 ± 15.7	43.6 ± 12.7
	α9 TCL	9564	2487	119.9 ± 22	119.9 ± 22	0 ± 0	0 ± 0	4.2 ± 4.1
Untransformed cells		34935	17451	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Appendix 5.3.2: Frequencies of foci ± standard error (S.E.) produced on the mixed monolayers. The values are given for individual cell lines of all foci produced (irrespective of category) as well as values for the frequencies of foci of each category produced on the mixed monolayers of C3H10T½ cells. STR is the abbreviation for the spontaneous cells, α denotes alpha-particle induced foci, X denotes X-ray induced foci, TCL is the abbreviation for tumour cell lines. Combined data are presented in figures 5.3.3 to 5.3.8.

Total Number of Foci						
Focus Type	Cell Line	All Foci	(+) Foci	(X/+) Foci	(X) Foci	(-) Foci
Total Number of Foci produced on Confluent Monolayers (± S.E.)						
(+)	X-ray	1479 ± 38	1007 ± 32	207 ± 14	41 ± 6	224 ± 15
	Alpha-particles	772 ± 26	241 ± 15	131 ± 11	148 ± 12	252 ± 13
	STR	14 ± 4	9 ± 3	0 ± 0	0 ± 0	5 ± 2
(+) TCL	X-ray	3396 ± 58	465 ± 22	707 ± 27	1934 ± 44	290 ± 17
	Alpha-particles	702 ± 27	690 ± 26	6 ± 2	1 ± 1	5 ± 2
	STR	510 ± 23	55 ± 7	59 ± 8	390 ± 20	6 ± 2
(X/+)	X-ray	19 ± 4	8 ± 3	5 ± 2	2 ± 1	4 ± 2
	Alpha-particles	308 ± 18	4 ± 2	2 ± 1	275 ± 17	47 ± 7
(X/+) TCL	X-ray	259 ± 16	82 ± 9	36 ± 6	104 ± 10	37 ± 6
	Alpha-particles	9 ± 3	3 ± 2	1 ± 1	1 ± 1	4 ± 2
(X)	X-ray	255 ± 16	1 ± 1	2 ± 1	241 ± 16	11 ± 3
(X) TCL	X-ray	866 ± 29	22 ± 5	0 ± 0	844 ± 29	0 ± 0
(-)	X-ray	25 ± 5	13 ± 4	0 ± 0	0 ± 0	12 ± 3
	Alpha-particles	9 ± 3	9 ± 3	0 ± 0	0 ± 0	0 ± 0
(-) TCL	X-ray	971 ± 31	918 ± 30	0 ± 0	0 ± 0	53 ± 7
	Alpha-particles	218 ± 15	209 ± 14	0 ± 0	0 ± 0	9 ± 3
Total Number of Foci produced on Mixed Monolayers (± S.E.)						
(+)	X-ray	940 ± 31	837 ± 29	7 ± 3	6 ± 3	90 ± 9
	Alpha-particles	576 ± 22	61 ± 7	210 ± 14	24 ± 5	281 ± 15
	STR	32 ± 6	13 ± 4	0 ± 0	0 ± 0	19 ± 4
(+) TCL	X-ray	3145 ± 56	796 ± 28	1158 ± 34	1161 ± 34	30 ± 6
	Alpha-particles	166 ± 13	152 ± 12	0 ± 0	0 ± 0	14 ± 4
	STR	168 ± 13	39 ± 6	4 ± 2	7 ± 3	118 ± 11

Total Number of Foci						
Focus Type	Cell Line	All Foci	(+) Foci	(X/+) Foci	(X) Foci	(-) Foci
(X/+)	X-ray	8 ± 3	3 ± 2	1 ± 1	0 ± 0	4 ± 2
	Alpha-particles	893 ± 30	7 ± 3	20 ± 4	841 ± 29	25 ± 5
(X/+) TCL	X-ray	117 ± 11	39 ± 6	0 ± 0	1 ± 1	77 ± 9
	Alpha-particles	66 ± 8	2 ± 1	0 ± 0	3 ± 2	61 ± 8
(X)	X-ray	243 ± 16	1 ± 1	0 ± 0	238 ± 15	4 ± 2
(X) TCL	X-ray	756 ± 28	0 ± 0	27 ± 5	729 ± 27	0 ± 0
(-)	X-ray	9 ± 3	0 ± 0	0 ± 0	0 ± 0	9 ± 3
	Alpha-particles	72 ± 8	14 ± 4	0 ± 0	0 ± 0	58 ± 8
(-) TCL	X-ray	827 ± 29	149 ± 12	0 ± 0	529 ± 23	149 ± 12
	Alpha-particles	41 ± 6	40 ± 6	0 ± 0	0 ± 0	1 ± 1

Appendix 5.3.3: Total number of foci ± standard error (S.E.) produced on the confluent and mixed monolayers. The values are given for the different categories of foci or tumour cells (combined data of a number of cell lines) of all foci produced (irrespective of category) as well as values for the number of foci of each category produced on the confluent and mixed monolayers of C3H10T½ cells. STR is the abbreviation for the spontaneous cells, TCL is the abbreviation for tumour cell lines. These data are presented in figures 5.3.9 to 5.3.13.

Section 5.4.

Growth Characteristics *in vitro*

List of figures

It has been stated already in this thesis that the transformation of cells *in vitro* involves numerous changes within these cells. The phenotypic properties of transformed cells include altered cellular morphology and multicellular organisation into colonies and foci, ability to grow in semi-solid medium and the ability to produce tumours in nude mice. In addition, the ability to reach increased cell density levels *in vitro* before slowing or stopping of cell proliferation in confluent cultures and the ability to grow in culture medium with little or no serum supplement has been documented for transformed cells (Smets 1980, Borek 1985).

In this section the emphasis is on the growth of the focus and tumour cells *in vitro*. The growth parameters examined are referred to as the lag time, doubling time and saturation density. Here, the lag time is defined as the time taken for the initial number of cells seeded in the assay to be doubled. Doubling time is the time taken for the population of cells to be doubled when the cells are in the exponential phase of growth. The definition of the saturation density is that used by Reznikoff *et al.* (1973) which is the maximum cell density attained under specified culture conditions. Saturation density is usually higher than the confluent cell density which is the cell number which forms a smooth continuous monolayer of cells (Reznikoff *et al.* 1973).

Experimental details and calculation of growth parameters

The details for the assay to determine the growth curves of these cells are given in chapter two. Briefly the cells were seeded into 35mm culture dishes containing growth medium at approximately six thousand cells per dish, and replicate dishes counted daily for fourteen days. Duplicate assays were set-up for each cell line. Similar assays were set-up for the untransformed control C3H10T½ cells with each batch of focus or tumour cells examined. The number of cells per dish was plotted on a logarithmic scale versus time on a linear scale. Figure 5.4.1 presents a typical example of a growth curve, indicating how the lag time, doubling time and saturation density are determined. The line fitted to the growth curve was fitted using the formula:

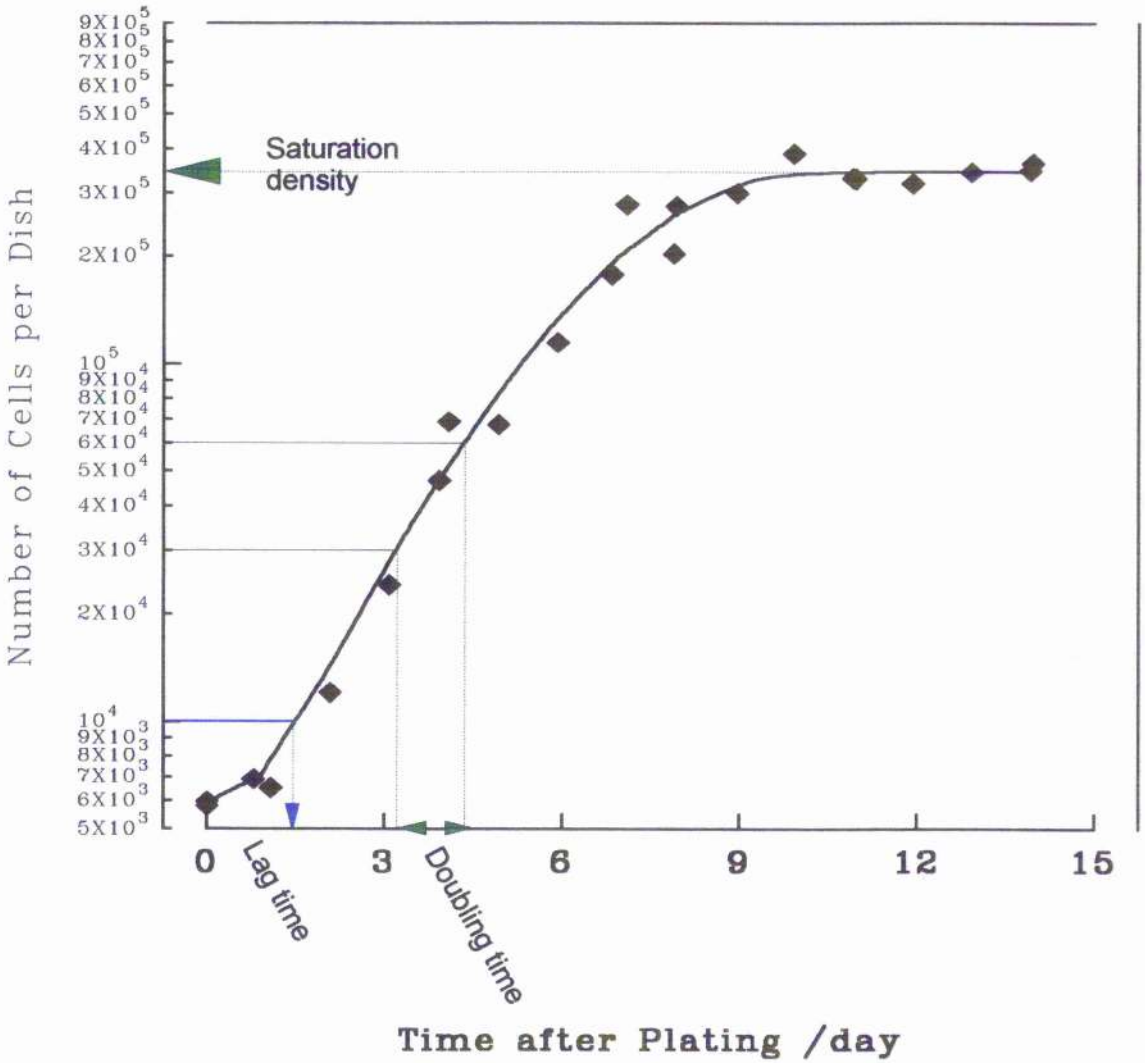


Figure 5.4.1. Sample growth curve. The number of cells present in 35mm petri dishes was calculated and plotted against time. The line fitted to the data using the equation detailed in the text is also shown.

$$N=N_0+C(1-e^{a(1-e^{bt})+abt})$$

N = number of cells per dish

t = time (days)

N_0 = number of cells initially seeded per dish

a = constant related to the initial slope of the graph

b = constant related to the exponential phase of the graph (day^{-1})

c = constant related to the plateau phase of the graph (saturation density)

The determination of the doubling time and lag phase was done by double differentiation of the above formula and then numerically solving the equations using the constants N_0, a, b and c[1]. (formula noted in Little and Charles 1991, original reference Makeham 1860).

The standard culture medium for C3H10T½ cells is Eagle's basal medium supplemented with ten percent heat inactivated foetal calf serum, L - glutamine and antibiotics (optional) (Reznikoff *et al.* 1973). One of the reported properties of transformed cells is the ability to grow in medium supplemented with lower levels of serum than routinely required for untransformed cells (Smets 1980, Borek 1985). Thus it was decided to examine the growth characteristics of focus and tumour cells grown using the standard medium supplement of ten percent serum and the reduced medium supplement of five percent serum.

Comparison of the lag times, doubling times and saturation densities are presented in figures 5.4.2 to 5.4.4 (a and b). Each figure presents the data for growth using standard serum - supplemented medium (10%) and reduced serum - supplemented medium (5%). In each set of figures the first figure (a) compares the untransformed C3H10T½ cells and the X-ray and alpha-particle induced foci (combined data on tumourigenic and non - tumourigenic foci) while the second figure (b) in each set presents comparison of X-ray and alpha-particle induced foci and the corresponding tumour cells with the data from the foci including only the tumourigenic foci. Data presented in figures 5.4.2 to 5.4.4 are presented in the appendices as well as the data for the individual focus and tumour cell lines.

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[1]mathematics kindly provided by Paul Grimwood

Lag times

Figure 5.4.2 (a) presents the lag time of the various categories of X-ray and alpha-particle induced foci (combined data of tumourigenic and non - tumourigenic foci). No differences are noted between growth in standard serum - supplemented medium and reduced serum - supplemented culture medium with the exception of a longer lag time for the spontaneous (+) focus and the alpha-particle induced (X) focus in the reduced serum conditions (one cell line each examined). In each of the focus categories no major differences are found between X-rays and alpha-particles although the mean lag times are shorter for the alpha-particle induced (X/+) and (-) foci than for the corresponding X-ray induced foci.

Figure 5.4.2 (b) compares the lag times of X-ray and alpha-particle induced foci and tumour cells when the cells were grown using standard serum-supplemented culture medium and reduced serum - supplemented medium. The only clear difference between growth in standard serum conditions and reduced serum conditions is for the alpha-particle induced (X/+) foci where the reduced serum conditions resulted in a longer lag time. Other differences (which are not significant) between the standard and reduced growth conditions are longer mean lag times for the X-ray induced (X/+) and (-) foci when grown in reduced serum - supplemented culture medium. Comparison of the different focus categories presents no differences between X-rays and alpha-particles for the (+) or (X/+) foci nor for their corresponding tumour cells. The only focus category which shows a little difference between X-rays and alpha-particles is the (-) foci and their tumour cells where a longer lag time exists for the X-ray induced foci and tumour cells for growth in standard and reduced serum conditions (difference in the reduced serum conditions for the foci not as pronounced as for the tumour cells). No differences are noted between foci and their corresponding tumour cells with the exception of a longer lag time for the tumour cells from the (X) focus (one cell line examined). Ratios were calculated of lag time (standard conditions) to lag time (reduced conditions) for the individual focus and tumour cell lines. These ratios were then combined to get a mean ratio for each group of foci or tumour cells (ratios shown in appendix 5.4.6). Differences between groups of cell lines were tested for significance using the student's *t*-test and the variance - ratio test (F-test). The only

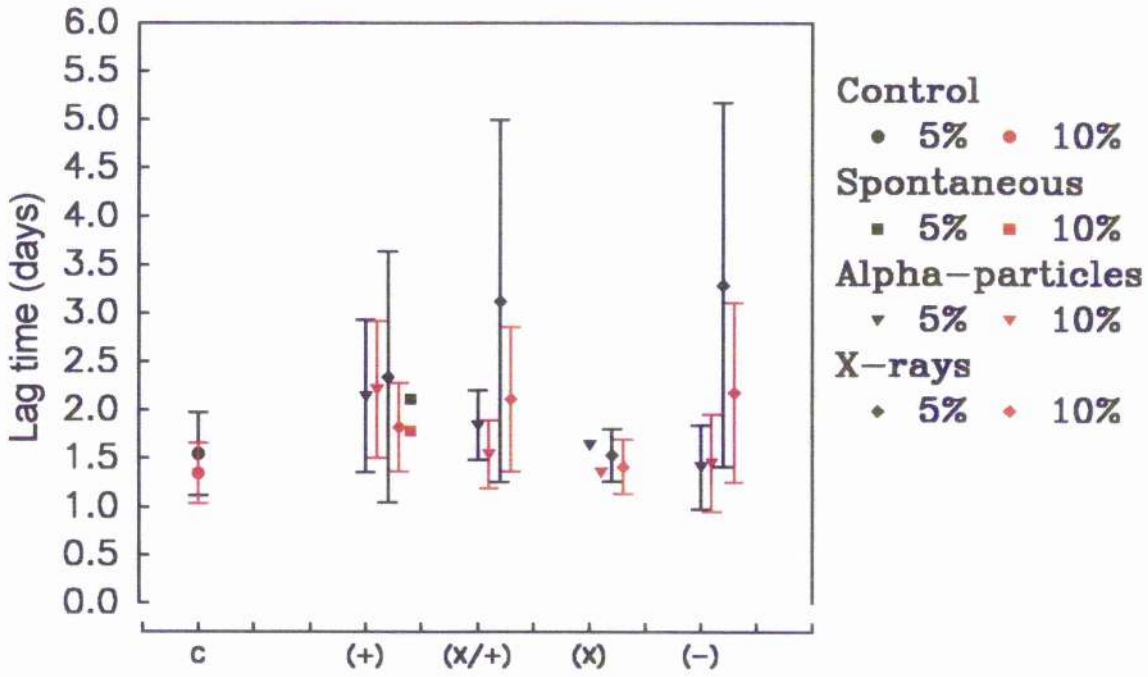

Figure 5.4.2 (a). Lag times of the various categories of foci and the untransformed C3H10T½ cells (C/control(n=12)). Lag time is defined as the time taken for the population of cells to reach double the initial number of cells seeded. Data are shown for standard serum - supplemented growth medium (10%) and reduced serum - supplemented medium (5%). The number of foci examined (n=X-ray induced foci, alpha - particle induced foci) was as follows: (+) n=9, 11; (X/+) n=4, 5; (X) n=2, 1; (-) n=5, 5. Data on one spontaneous focus (+) are also presented. Data include both tumourigenic and non - tumourigenic foci.

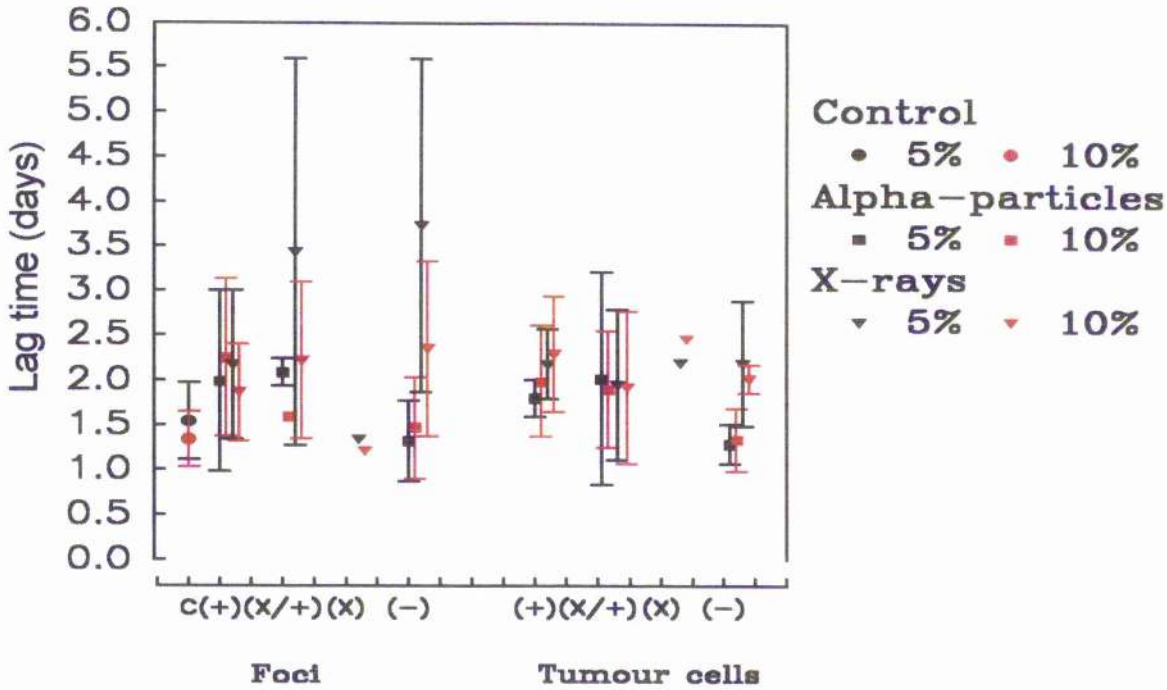

Figure 5.4.2 (b). Lag times of the various categories of foci and tumour cells and the untransformed C3H10T½ cells (C/control (n=12)). Lag time is defined as the time taken for the population of cells to reach double the initial number of cells seeded. Data are shown for standard serum - supplemented growth medium (10%) and reduced serum - supplemented medium (5%). The number of foci / tumour cells examined (n=X-ray induced foci, alpha - particle induced foci) was as follows: (+) n=6, 6; (X/+) n=3, 2; (X) n=1, 0; (-) n=4, 4. Data on one spontaneous focus (+) are also presented. Data on the foci include only the tumourigenic foci.

significant differences ($0.01 < p < 0.05$) found using these ratios is between the X-ray induced (X/+) foci and their corresponding tumour cells and between the X-ray and alpha-particle induced (-) foci.

Doubling times

Figure 5.4.3 (a) shows the doubling times of the untransformed, spontaneous, X-ray and alpha-particle induced foci grown in standard serum - supplemented growth medium and reduced serum - supplemented medium. No differences were observed between growth in standard serum - supplemented medium and reduced serum - supplemented culture medium. In each of the focus categories no major differences are found between X-rays and alpha-particles although the mean doubling times are shorter for the alpha-particle induced (X/+) and (-) foci for growth in both standard and reduced serum conditions and for the (+) foci grown in reduced serum conditions than for the corresponding X-ray induced foci.

Figure 5.4.3 (b) compares the doubling times of X-ray and alpha-particle induced foci and their corresponding tumour cells when the cells were grown using standard serum-supplemented culture medium and reduced serum - supplemented medium. As for the lag time the only apparent difference between growth in standard serum conditions and reduced serum conditions is for the alpha-particle induced (X/+) foci where the reduced serum conditions resulted in a longer doubling time and the tumour cells from the (X) foci where the reverse was observed (only one tumour cell line was examined). Other differences (which are not significant) between the standard and reduced growth conditions are longer mean doubling times for X-ray induced (+) foci and tumour cells from the X-ray and alpha-particle induced (X/+) foci when grown in reduced serum - supplemented culture medium. Comparison of the different focus categories displays no great differences between X-rays and alpha-particles for the (+) or (X/+) foci nor for their corresponding tumour cells. The only focus category which shows a difference is the (-) foci where the X-ray induced foci have a longer doubling time when grown in standard and reduced serum conditions than the alpha-particle induced foci. No differences are apparent for the doubling times of the tumour cells from the (-) foci between X-rays and alpha-particles. When ratios of the doubling

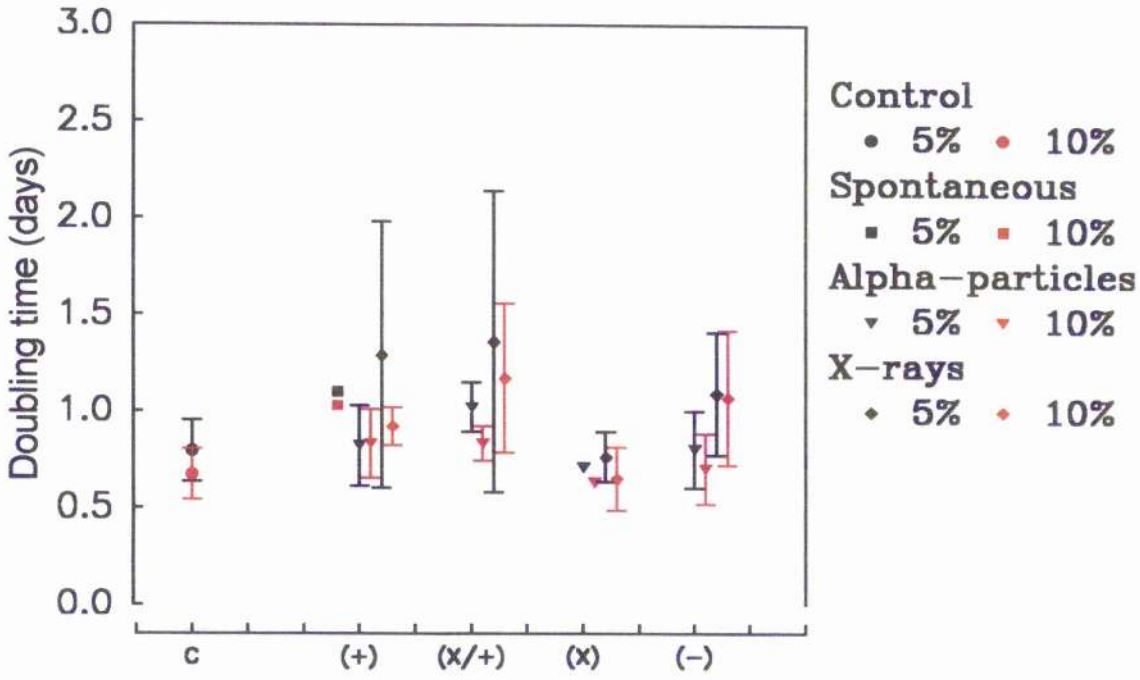

Figure 5.4.3 (a). Doubling times of the various categories of foci and the untransformed C3H10T½ cells (C/control (n=12)). Doubling time is defined as the time taken for the population of cells to double when the cells are in the exponential phase of growth. Data are shown for standard serum - supplemented growth medium (10%) and reduced serum - supplemented medium (5%). The number of foci examined (n=X-ray induced foci, alpha - particle induced foci) was as follows: (+) n=9, 11; (X/+) n=4, 5; (X) n=2, 1; (-) n=5, 5. Data on one spontaneous focus (+) are also presented. Data include both tumourigenic and non - tumourigenic foci.

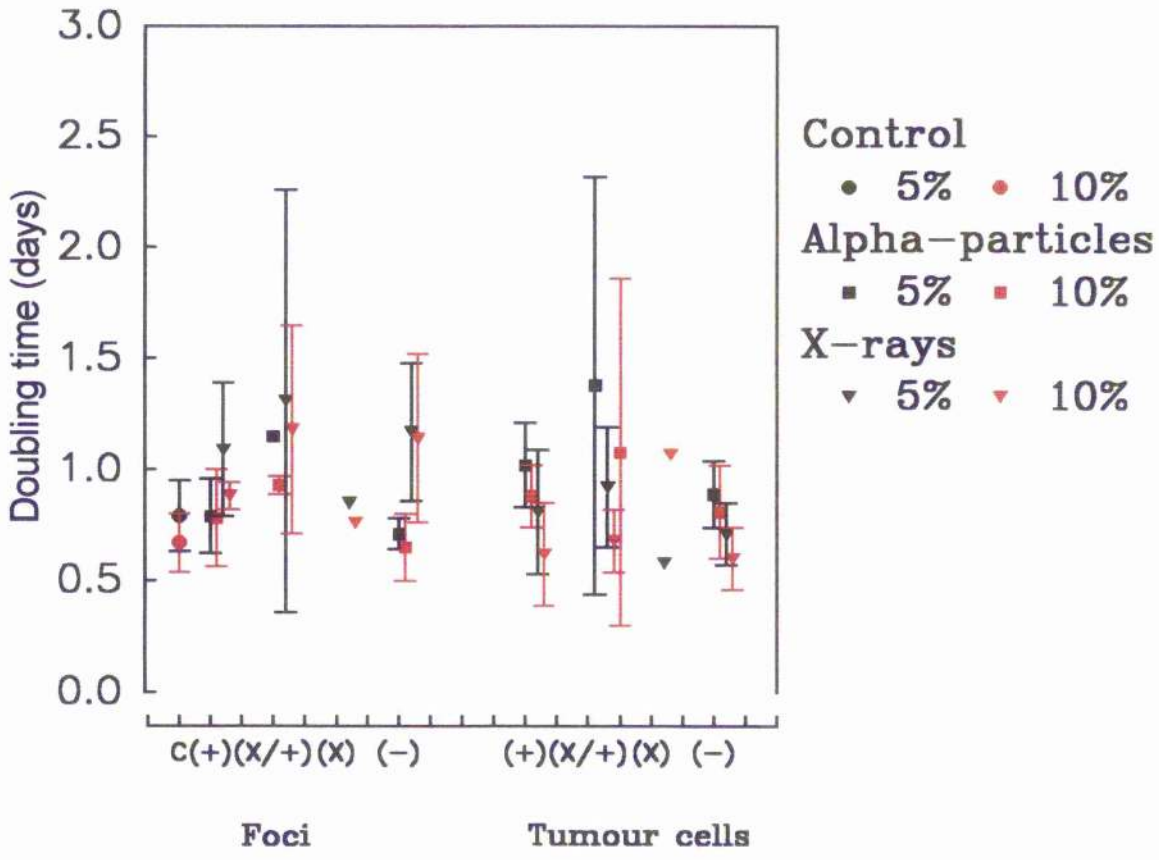

Figure 5.4.3 (b). Doubling times of the various categories of foci and tumour cells and the untransformed C3H10T½ cells (C/control (n=12)). Doubling time is defined as the time taken for the population of cells to double when the cells are in the exponential phase of growth. Data are shown for standard serum - supplemented growth medium (10%) and reduced serum - supplemented medium (5%). The number of foci / tumour cells examined (n=X-ray induced foci, alpha - particle induced foci) was as follows: (+) n=6, 6; (X/+) n=3, 2; (X) n=1, 0; (-) n=4, 4. Data on one spontaneous focus (+) are also presented. Data on the foci include only the tumourigenic foci.

times (standard: reduced conditions) were calculated and examined as described for the lag times the only significant differences found ($0.01 < p < 0.05$) are between the (+) and (X/+) foci induced by alpha-particles and between X-rays and alpha-particles for (+) foci.

Saturation densities

Figure 5.4.4 (a) shows that the saturation densities of the untransformed C3H10T½ cells, spontaneous, X-ray and alpha-particle induced foci (combined data of tumourigenic and non - tumourigenic foci) are higher in all cases when the cells were grown in standard serum - supplemented growth medium rather than reduced serum - supplemented medium for all categories of foci. Comparison of the saturation densities when the foci were grown in standard conditions shows that, while most X-ray and alpha-particle induced foci have similar or slightly lower saturation densities to the untransformed cells there are three exceptions. Saturation densities of the alpha-particle induced (+) and (X) foci are lower while those of the alpha-particle induced (-) foci are higher than the density of the untransformed cells. Comparison of the saturation densities of the foci grown using the reduced serum - supplemented culture medium also shows most foci having similar or slightly lower densities to the untransformed cells with the lowest saturation densities presented by the X-ray induced (X/+) foci and the alpha-particle induced (X) foci. Comparison of the different categories of alpha-particle induced foci shows the highest saturation density by the (-) foci with approximately equal densities by the (X) and (+) foci and the saturation density of the (X/+) foci in the middle of this range under standard and reduced serum conditions. Comparison of the X-ray induced foci shows similar densities for the different categories (with a slightly higher density by the (+) foci) except for the lower density of the (X/+) foci mentioned above. Examination of the (+) foci shows the spontaneous and X-ray induced foci have higher densities than the alpha-particle induced foci for growth in both standard and reduced growth conditions, the difference being more pronounced in the standard conditions. No major differences between radiation types is observed for the (X/+) foci grown in standard conditions while the alpha-particle induced foci show higher saturation densities in the reduced serum

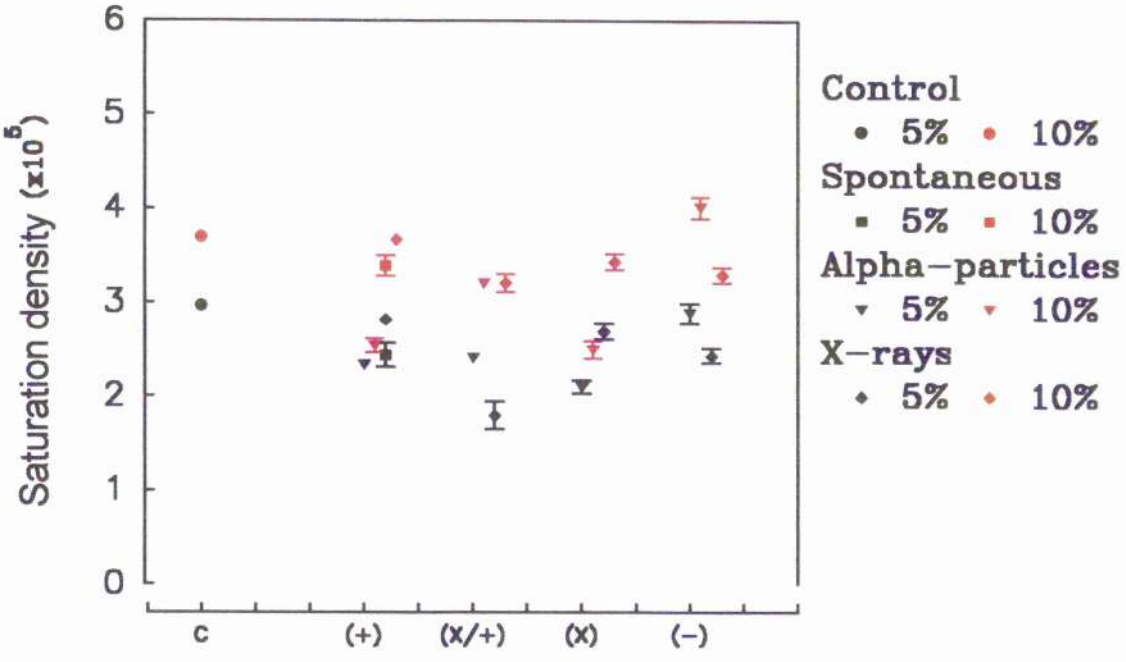

Figure 5.4.4 (a). Saturation densities of the various categories of foci and the untransformed C3H10T½ cells (C/control (n=12)). Saturation density is defined as the maximum cell density attainable under specified growth conditions. Data are shown for standard serum - supplemented growth medium (10%) and reduced serum - supplemented medium (5%). The number of foci examined (n=X-ray induced foci, alpha - particle induced foci) was as follows: (+) n=9, 11; (X/+) n=4, 5; (X) n=2, 1; (-) n=5, 5. Data on one spontaneous focus (+) are also presented. Data include both tumourigenic and non - tumourigenic foci.

conditions than the X-ray induced counterparts. Examination of the (X) foci shows a higher saturation density for X-ray induced foci for growth in both standard and reduced serum - supplemented culture medium, while the (-) foci show the reverse with higher saturation densities evident for the alpha-particle induced foci.

Figure 5.4.4 (b) compares the saturation densities of the X-ray and alpha-particle induced foci (data of tumourigenic foci only) and their corresponding tumour cells when the cells were grown in both standard and reduced serum - supplemented growth medium. Saturation densities of all foci and tumour cells with the sole exception of the tumour cells from the (X) foci (only one cell line examined) is higher for cells grown in standard serum -supplemented culture medium than for those grown in reduced serum - supplemented medium. X-ray induced (+) and (X) foci have higher saturation densities than the untransformed cells when the cells were grown in standard serum - supplemented culture medium and only the (X) foci have higher densities than the untransformed cells in the reduced serum growth conditions. X-ray induced (X/+) foci and alpha-particle induced (-) foci have similar saturation densities to the untransformed cells while the remainder of the foci have lower densities when grown in standard growth conditions. Growth of cells in culture medium with reduced serum levels produced similar saturation densities to the untransformed cells for the X-ray induced (+) foci and the alpha-particle induced (-) foci while the remainder of the foci have lower saturation densities. No alpha-particle induced (X) focus or tumour cells were examined. Comparison of the different focus categories shows the X-ray induced (+) and (X) foci produced the highest saturation densities while the alpha-particle induced (+) foci produced the lowest densities in the standard growth conditions. For the reduced growth conditions the X-ray induced (X) foci produced the highest saturation densities. Of the X-ray induced foci the (X) foci had the highest saturation densities while of the alpha-particle induced foci the highest saturation density was produced by the (-) foci in both standard and reduced growth conditions.

Examination of the tumour cells reveal fewer differences than observed for the foci. Growth in standard growth conditions resulted in higher saturation densities for all tumour cells except those from the alpha-particle induced (+) foci (no difference seen between the saturation densities) and the X-ray induced (X) foci. Comparison of

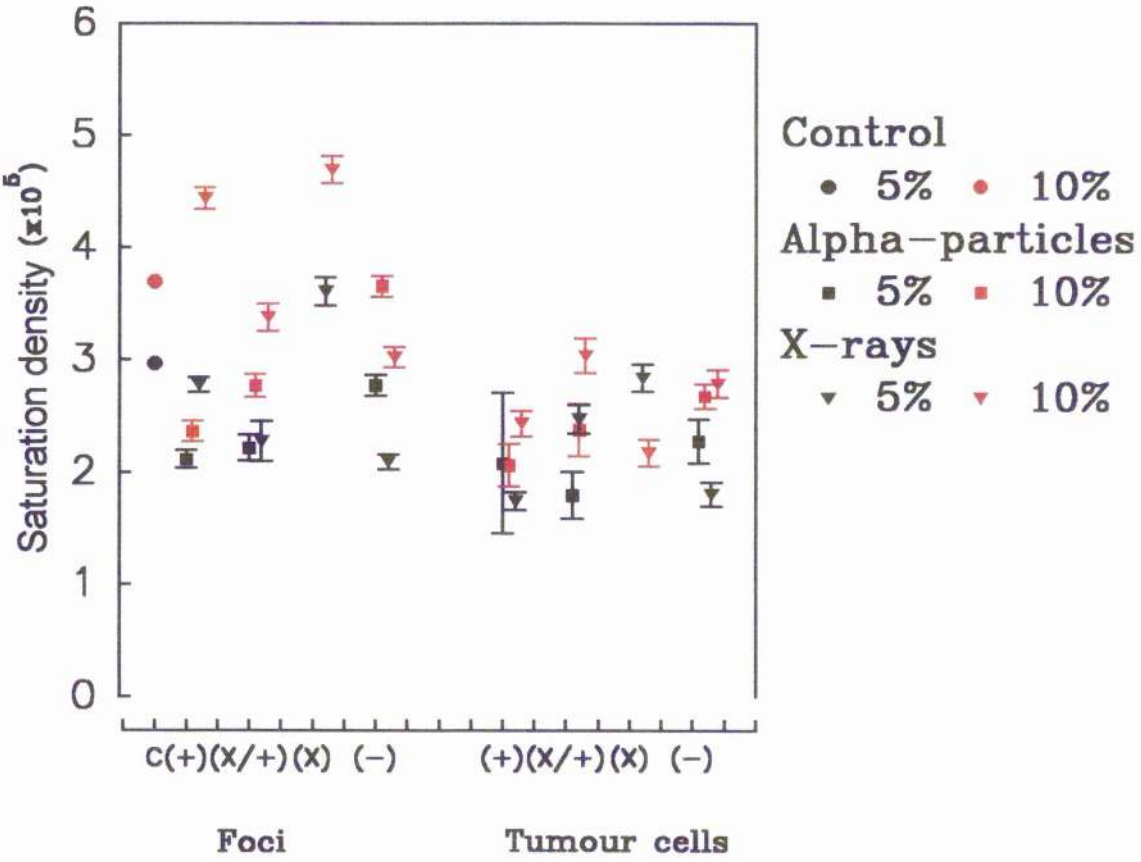

Figure 5.4.4 (b). Saturation densities of the various categories of foci and tumour cells and the untransformed C3H10T½ cells (C/control (n=12)). Saturation density is defined as the maximum cell density attainable under specified growth conditions. Data are shown for standard serum - supplemented growth medium (10%) and reduced serum - supplemented medium (5%). The number of foci / tumour cells examined (n=X-ray induced foci, alpha - particle induced foci) was as follows: (+) n=6, 6; (X/+) n=3, 2; (X) n=1, 0; (-) n=4, 4. Data on one spontaneous focus (+) are also presented. Data on the foci include only the tumourigenic foci.

the tumour cells from the different focus categories shows X-ray induced (X/+) and (X) cells produced the highest saturation densities in the standard growth conditions and reduced growth conditions respectively. Examination of the tumour cells from the (+) foci shows no great difference between X-rays and alpha-particles although the X-ray induced foci have a higher saturation density than the alpha-particle induced foci for growth in standard growth conditions, no difference is found in the reduced growth conditions. Tumour cells from (X/+) foci show higher saturation densities for X-rays than for alpha-particles for cells grown in both types of growth conditions. No differences are observed between X-rays and alpha-particles for the tumour cells from the (-) foci grown in standard growth conditions whereas in the reduced growth conditions the alpha-particle induced cells produced higher saturation densities.

Comparison of the foci and their corresponding tumour cells show the X-ray induced (+) foci produced higher saturation densities than the tumour cells while the alpha-particle induced (+) and (X/+) foci show little difference between foci and tumour cells. X-ray induced (X/+) foci produced higher saturation densities in standard growth conditions and slightly lower densities in reduced growth conditions when compared with the tumour cells. X-ray induced (X) foci produced higher saturation densities than the tumour cells with the differences more pronounced in standard growth conditions. Both X-ray and alpha-particle induced (-) foci produced higher saturation densities than the tumour cells although the difference for the X-rays is not as pronounced as for the other focus categories. When ratios of saturation density (standard: reduced conditions) were examined as described for the lag and doubling times the significant differences noted ($0.01 < p < 0.05$) are between X-rays and alpha-particles for (+) foci and tumour cells from (+) foci.

Discussion

Lag and doubling times

No substantial differences were found between the different cell lines for either the lag time or doubling time of the cells. The lag time as defined in this section is the time taken for the cells to double the initial number of cells seeded in the culture dishes and this time was generally found to be one to two days. The doubling time is the time taken for the population of cells to double its number when the cells are in the exponential phase of growth, this generally takes sixteen to twenty hours. After examining the different focus and tumour cell categories with respect to each other and to the untransformed cells, as well as by radiation type and different growth conditions only a few differences in lag times were noted. Alpha-particle induced (X/+) foci had longer lag times when grown in the reduced serum - supplemented culture medium than in standard conditions and the X-ray induced (-) foci and tumour cells showed longer lag times than the corresponding alpha-particle treated cells in both types of growth conditions (although the difference for the foci grown in the reduced serum - supplemented culture medium was not as pronounced as for the tumour cells). Examination of the same parameters for the doubling time revealed the same observation for the alpha-particle induced (X/+) foci which had a longer doubling time when grown in the reduced serum - supplemented culture medium and for the X-ray induced (-) foci which showed a longer doubling time than the alpha-particle induced equivalent in both growth conditions (no difference between X-rays and alpha-particles for the tumour cells from the (-) foci).

Saturation densities

Most of the differences between cell lines appeared in the saturation densities, the maximum cell density the cells attained *in vitro*. Generally the saturation density was higher for cells grown in standard growth conditions than for cells grown in reduced serum levels. The only two exceptions to this were by tumour cells, those from the alpha-particle induced (+) foci where no difference in saturation densities was noted, and those from the X-ray induced (X) foci where the saturation density was higher when the growth conditions involved lower serum levels than routinely used.

While many of the foci had comparable or slightly lower saturation densities than the untransformed cells, the tumour cells consistently had lower densities than the untransformed cells, with the same pattern observed for growth in standard and reduced serum conditions. The radiation induced foci (combined data of tumourigenic and non - tumourigenic foci) all had similar or lower saturation densities than the untransformed cells. The highest saturation density for the alpha-particle induced foci was produced by the (-) foci and the lowest density shared by the (X) and (+) foci. Similar densities are noted for the different categories of X-ray induced foci except notably lower densities by the (X/+) foci in the reduced serum conditions. Examination of each focus category on its own shows the highest saturation density for the (+) foci was produced by the spontaneous and X-ray induced foci in both growth conditions (difference more pronounced in the standard conditions) while no differences were noted for the (X/+) foci grown in standard conditions and the alpha-particle induced foci produced higher saturation densities in reduced growth conditions. The highest saturation densities for the (X) foci were produced by the X-ray induced foci, while the alpha-particles produced the highest density for the (-) foci in both growth conditions. X-ray induced (-) foci had longer lag and doubling times in both growth conditions than the alpha-particle equivalent which contributed to the difference between radiation types in this focus category.

The remaining comparison of saturation density is between radiation induced foci (data on tumourigenic foci only) and their tumour cells. No data are available for alpha-particle induced (X) foci or tumour cells. Examination of the foci grown in standard growth conditions shows the X-ray induced (+) and (X) foci produced higher saturation densities while the alpha-particle induced (+) and (X/+) and the X-ray induced (-) foci produced lower densities than the untransformed cells. The X-ray induced (X) foci grown in reduced serum levels had a higher saturation density, while the X-ray induced (+) and alpha-particle induced (-) foci had similar densities and the other categories had lower densities than the untransformed cells. Comparison of the focus categories to each other displays the highest saturation density by the X-ray induced (+) and (X) foci and the lowest density by the alpha-particle induced (+) foci in the standard growth conditions while in the reduced growth conditions the highest

saturation density was produced by the X-ray induced (X) foci. Of the alpha-particle induced foci the highest saturation density was of (-) focus cells while the highest density of the X-ray induced foci was produced by the (X) foci in both types of growth conditions. Examination of the tumour cells shows the X-ray induced (X/+) and (X) foci produced the highest saturation density in the standard and reduced serum conditions respectively. No differences between X-rays and alpha-particles were observed in tumour cells from the (+) foci grown in reduced serum - supplemented culture medium while a higher saturation density was produced by the X-ray induced cells in the standard conditions. Tumour cells from the X-ray induced (X/+) foci produced higher saturation densities than the alpha-particle equivalent for both types of growth conditions while the tumour cells from the alpha-particle induced (-) foci produced higher densities than the X-ray equivalent (which showed longer lag times) for the reduced serum conditions and no difference was noted between X-rays and alpha-particles for the (-) tumour cells in the standard growth conditions. Comparison of the foci with their corresponding tumour cells presents that X-ray induced foci produced higher saturation densities than tumour cells for all categories in both growth conditions (except no difference in saturation density was observed for (X/+) cells in the reduced serum conditions). Differences were more pronounced in the standard growth conditions. The alpha-particle induced (+) and (X/+) foci showed no differences between foci and tumour cells in either growth conditions while the (-) foci produced higher saturation densities than the tumour cells for both types of growth conditions. The most consistent pattern between X-rays and alpha-particles is that for foci (either combined as tumourigenic and non - tumourigenic or left as tumourigenic only) and tumour cells, examination of the saturation densities reveals that where differences occur the X-ray treated cells show higher saturation densities for the (+), (X/+) and (X) focus categories whereas in all cases the alpha-particle treated cells produced higher densities for the (-) focus category.

Comparison with data of other authors

Most authors using the C3H10T½ cell line report the doubling time of the untransformed cells in the range of fifteen to nineteen hours and the saturation density

as approximately 3 $x10^4$ cells / cm^2 (for example Reznikoff *et al.* 1973, Male *et al.* 1987, Narayan *et al.* 1984, Bettega *et al.* 1989 and Smith *et al.* 1993). Data presented in this thesis compare favourably with these values (average doubling time of sixteen hours and a saturation density of 3.6 $x10^4$ cells / cm^2). Studies which examined the growth parameters of doubling time and saturation densities for transformed C3H10T½ cells indicated a longer doubling time in some cases (Reznikoff *et al.* 1973, Smith *et al.* 1993) and no difference in doubling time in others (Male *et al.* 1987, Narayan *et al.* 1984), while most studies reported higher saturation densities for transformed cells (Reznikoff *et al.* 1973, Male *et al.* 1987, Smith *et al.* 1993) with one study (Narayan *et al.* 1984) indicating no difference in saturation density between transformed and untransformed cells. These studies examined transformed C3H10T½ focus cells only, no tumour cells were examined.

Few studies report data on altering the growth conditions of transformed and untransformed C3H10T½ cells to investigate if transformed cells require fewer growth factors than untransformed cells as has been reported (Smets 1980, Borek 1985). One study examined the growth *in vitro* of tumour cells isolated from neoplasms produced by C3H10T½ cells implanted subcutaneously in C3H mice, attached to 1 x 5 x 10mm plastic plates (Paranjpe *et al.* 1978). Examination of four of these tumour cell lines showed similar growth rates of the cells in standard growth conditions. However when the serum supplement to the growth medium was reduced to one percent or removed altogether the growth rate of all the tumour cells was markedly greater than the untransformed cells though less than the growth rate in standard conditions (Paranjpe *et al.* 1978). Furthermore there were no great differences between the tumour cell lines grown in the medium supplemented with one percent serum, while one of the cell lines showed a much longer latent period before exponential growth occurred when the cells were grown in culture medium containing no serum (Paranjpe *et al.* 1978).

In an earlier study by Bertram (1977) the growth of transformed C3H10T½ cells (not tumour cells as used in the above study) was examined, altering the serum content in the growth conditions. Changing the serum level from two percent to twenty percent had no effect on the growth of either the transformed or untransformed cells with the doubling time remaining about sixteen hours in all situations examined. The

only difference noted for the transformed cells agreed with the data of Reznikoff *et al.* (1973) for untransformed cells where an increased saturation density was noted in the higher serum levels (Bertram 1977). In the study of the influence of altering the serum content of the culture medium on the saturation density of untransformed cells by Reznikoff *et al.* (1973) the cultures were kept for six weeks without subculture after reaching confluence and the culture medium changed weekly or twice weekly with the medium supplemented with either five or ten percent serum. The only difference in saturation density found was an increased density of cells grown in culture medium supplemented with ten percent serum which received changes of medium twice weekly, indicating the untransformed C3H10T½ cells do respond to increased nutrients in the environment and reach a higher saturation density (Reznikoff *et al.* 1973).

Similar results were found in the data presented in this chapter where higher saturation densities were found in almost all cases for cells grown in standard (ten percent) serum - supplemented culture medium than in reduced (five percent) serum - supplemented medium. It has already been stated that supplying the cells with a fresh supply of nutrients during the course of the assay to determine the saturation density results in a higher cell density being reached. Under these conditions higher saturation densities have been observed for transformed cells (few studies examined tumour cells) compared to untransformed cells (for example Reznikoff *et al.* 1973, Male *et al.* 1987, Smith *et al.* 1993). Data presented in this thesis were obtained from an assay during which the cells did not receive any fresh supply of nutrients. As the saturation density is reached towards the end of the assay this may coincide with the supply of some nutrients beginning to reach critically low levels and although the cells reach a constant cell density, a fresh supply of nutrients might have encouraged the cells to reach a higher cell density. This could explain the lower saturation densities reached by the transformed cells in this thesis compared to other publications.

Tumour versus focus cells

Tumour cells consistently produced lower saturation densities than the untransformed cells while the focus cells produced similar or slightly lower densities

to the untransformed cells. It should be noted that the focus cell populations are more likely to be a heterogeneous collection of cells than the tumour cell population, containing a mix of focus and possibly untransformed cells while it is likely that the tumour cell population contains a relatively pure population of tumour cells. Thus conclusively transformed cells, that is tumour cells (the ultimate test of transformed cells *in vitro* is the ability to produce tumours *in vivo*) actually have a lower saturation density than untransformed C3H10T½ cells under the conditions of the assay used in this thesis. The absence of a difference between either the lag times or the doubling times of the tumour cells and the untransformed cells implies both cell populations should take approximately the same time to reach their saturation densities. There are two possible explanations for the lower saturation densities of the tumour cells, firstly, that tumour cells have a greater surface area on the culture dishes than the untransformed cells therefore one would expect fewer tumour cells per unit area or secondly that the tumour cells are less well attached to the plastic culture surface and detach easily into the culture medium (especially if the nutrient levels are low) and thus become excluded from the cell counts. The second possibility seems the most likely scenario and is supported by observations made during the focus reconstruction assays (section 5.3.) where poor plating efficiencies (carried out on plastic culture flasks) for some tumour cells were observed yet the same cells grew well and produced foci when they were seeded onto confluent monolayers of untransformed cells, indicating a poor affinity for the plastic culture surface rather than low viability of the cells, a phenomenon not observed in the focus cells.

The data presented in this section are discussed further in conjunction with the tumourigenicity and focus reconstruction data already presented in previous sections and with the cytogenetics data presented in the next section in an overview in section 5.6.

Summary

Data presented in this section generally showed no differences in either the lag time (time taken for the cells to double the initial number of cells seeded) or doubling time (time taken for the population of cells to double its number in the exponential phase of growth) of the majority of the cell populations examined. This was the case irrespective of focus category or radiation type and was not influenced by the cells being transformed or tumourigenic in nature.

Most of the differences between cell lines appeared in the saturation densities, the maximum cell density the cells attained *in vitro*. Generally the saturation density was higher for cells grown in standard growth conditions than for cells grown in reduced serum levels. Most of the foci produced saturation densities which were comparable or slightly lower than those of the untransformed cells whereas the tumour cells consistently had lower densities than the untransformed cells irrespective of the serum levels in the cultures. Radiation induced (+), (X) and (-) foci all produced higher saturation densities than the corresponding tumour cells irrespective of growth conditions while the (X/+) foci showed the same density in the standard growth conditions and a lower density in the reduced growth conditions than the corresponding tumour cells. A relatively constant pattern between X-rays and alpha-particles which emerged for the saturation densities was that for foci (either combined as tumourigenic and non - tumourigenic or left as tumourigenic only) and tumour cells, where differences occurred, the X-ray treated cells showed higher saturation densities for the (+), (X/+) and (X) focus categories whereas in all cases the alpha-particle treated cells produced higher densities for the (-) focus category.

Appendices

		Standard serum supplement			Reduced serum supplement		
Cells	Score	Lag (days)	D.T (days)	Saturation density ± S.E. ($x10^5$)	Lag (days)	D.T. (days)	Saturation density ± S.E. ($x10^5$)
X-ray induced foci							
X1	+	1.81	0.78	3.846 ± 0.107	1.52	0.94	2.642 ± 0.127
X3	+	1.39	0.88	2.528 ± 0.216			
X5	+	1.95	1.05	2.582 ± 0.173	1.79	1.07	1.510 ± 0.138
X7	+?	2.15	0.9	3.418 ± 0.140			
X8	+	1.12	0.76	3.45 ± 0.198	1.36	0.89	3.179 ± 0.102
X9	+	1.59	0.86	2.414 ± 0.125	1.15	0.79	1.812 ± 0.209
X10	+	1.45	0.92	3.364 ± 0.252	2.88	1.63	1.171 ± 0.164
X13	+	2.34	1.04	4.582 ± 0.313			
X15	+	2.16	1.09	1.801 ± 0.064	1.84	1.24	1.618 ± 0.121
X16	+?	1.62	0.95	3.619 ± 0.180			
X19	+	1.7	0.95	7.058 ± 0.318	2.27	1.24	4.66 ± 0.195
X22	+				5.41	3.14	5.228 ± 0.357
X23	+	1.6	0.96	2.744 ± 0.256			
X24	+	2.94	0.86	5.799 ± 0.311	3.35	0.93	3.592 ± 0.156
X27	+	1.65	0.88	4.155 ± 0.177	1.86	1.05	2.777 ± 0.093
X4	X/+	1.49	0.8	3.978 ± 0.192	1.62	0.73	2.998 ± 0.283
X12	X/+	1.77	1.11	2.694 ± 0.114	2.2	1.5	1.426 ± 0.26
X17	X/+	3.19	1.71	3.139 ± 0.285	5.82	2.41	1.207 ± 0.416
X20	X/+	1.99	1.04	3.02 ± 0.133	2.84	0.8	2.619 ± 0.168
X2	X	1.21	0.76	4.695 ± 0.122	1.34	0.85	3.607 ± 0.126
X11	X	1.6	0.53	2.173 ± 0.120	1.72	0.66	1.778 ± 0.118
X6	-?	1.49	0.83	4.391 ± 0.187	1.55	0.77	3.77 ± 0.281
X14	-?	1.55	0.9	3.04 ± 0.147	2.23	1.04	2.254 ± 0.145
X18	-	3.24	1.36	3.237 ± 0.178	5.13	1.27	1.927 ± 0.089
X21	-?	3.16	1.54	1.761 ± 0.146	5.53	1.54	0.935 ± 0.012
X25	-?	1.46	0.74	4.034 ± 0.235	2.02	0.81	3.255 ± 0.202

		Standard serum supplement			Reduced serum supplement		
Cells	Score	Lag (days)	D.T (days)	Saturation density ± S.E. ($x10^5$)	Lag (days)	D.T. (days)	Saturation density ± S.E. ($x10^5$)
Tumour cells from X-ray induced foci							
X1	+	2.05	0.56	2.646 ± 0.133	2.41	0.73	1.316 ± 0.153
X9	+	1.96	0.53	2.647 ± 0.356	1.62	0.77	1.312 ± 0.176
X10	+	2	0.46	2.392 ± 0.261	1.99	0.5	1.787 ± 0.148
X19	+	3.56	0.66	4.673 ± 0.269	2.75	1.12	2.622 ± 0.190
X22	+	1.27	0.84	2.846 ± 0.323	1.54	1.03	2.025 ± 0.204
X24	+	1.88	0.47	2.323 ± 0.374	2	0.57	1.511 ± 0.275
X27	+	2.27	1.06	2.274 ± 0.155	2.3	1.16	1.894 ± 0.186
X4	X/+	2.28	0.61	2.771 ± 0.120	2.17	1.19	2.187 ± 0.117
X17	X/+	0.95	0.59	3.478 ± 0.351	1.03	0.65	2.514 ± 0.272
X20	X/+	2.54	0.85	2.849 ± 0.273	2.66	0.92	2.707 ± 0.221
X2	X	2.2	0.58	2.836 ± 0.117	2.46	1.07	2.171 ± 0.118
X14	-?	2.03	0.49	2.394 ± 0.311	2.27	0.6	0.973 ± 0.159
X18	-	2.16	0.59	2.718 ± 0.152	2.93	0.63	1.399 ± 0.207
X21	-?	2.11	0.8	2.127 ± 0.167	2.3	0.91	1.886 ± 0.151
X25	-?	1.79	0.5	3.891 ± 0.304	1.25	0.69	2.955 ± 0.306

Appendix 5.4.1. Data on the individual X-ray induced foci and tumour cell lines. Table shows the lag time (lag), doubling time (D.T.) and saturation density (± standard error) of the cell lines. Data are combined and presented in appendices 5.4.3. to 5.4.5. Growth parameters were calculated for cells grown in culture medium supplemented with 10% foetal calf serum (standard) and with 5% foetal calf serum (reduced).

		Standard serum supplement			Reduced serum supplement		
Cells	Score	Lag (days)	D.T (days)	Saturation density ± S.E. ($x10^5$)	Lag (days)	D.T. (days)	Saturation density ± S.E. ($x10^5$)
Alpha-particle induced foci							
α1	+	2.23	1	3.287 ± 0.184	1.86	1.11	3.26 ± 0.108
α10	+	2.58	1.01	2.845 ± 0.293	2.94	1.08	2.239 ± 0.218
α11	+	2.85	1.1	2.137 ± 0.299	1.34	0.93	2.792 ± 0.332
α12	+	1.82	0.9	4.119 ± 0.248	2.09	0.96	2.562 ± 0.172
α16	+	3.76	0.45	2.093 ± 0.281	4.02	0.48	1.703 ± 0.189
α19	+	2.77	0.83	2.478 ± 0.384	2.07	0.59	2.263 ± 0.179
α20	+?	1.76	0.85	2.175 ± 0.139	1.56	0.88	2.004 ± 0.207
α26	+	1.42	0.71	3.588 ± 0.281	2.62	0.56	2.697 ± 0.095
α29	+	1.56	0.9	2.951 ± 0.126	1.52	0.94	2.548 ± 0.113
α32	+?	1.68	0.74	2.589 ± 0.216	1.67	0.8	1.987 ± 0.138
α46	+?	1.86	0.66	2.233 ± 0.162	1.81	0.73	1.648 ± 0.138
α2	X/+	1.6	0.95	2.389 ± 0.078	2.19	1.16	1.75 ± 0.01
α5	X/+	1.57	0.9	3.144 ± 0.193	1.98	1.14	2.684 ± 0.207
α13	X/+	2.07	0.78	2.773 ± 0.054	2.02	1	1.527 ± 0.059
α22	X/+	1.23	0.77	5.624 ± 0.169	1.74	0.93	4.333 ± 0.110
α31	X/+	1.23	0.73	2.079 ± 0.101	1.26	0.86	1.724 ± 0.137
α51	X?	1.35	0.63	2.493 ± 0.094	1.63	0.71	2.093 ± 0.068
α4	-	0.99	0.67	2.798 ± 0.155	1.1	0.76	2.742 ± 0.153
α24	-	1.37	0.91	5.423 ± 0.437	1.76	1.14	3.351 ± 0.371
α36	-?	2.29	0.43	2.738 ± 0.255	1.99	0.62	1.888 ± 0.206
α41	-	1.42	0.77	4.695 ± 0.122	1.07	0.71	3.099 ± 0.149
α55	-	1.18	0.72	4.391 ± 0.187	1.12	0.76	3.348 ± 0.237
Tumour cells from alpha-particle induced foci							
α10	+				1.78	1.29	0.524 ± 0.254
α11	+	1.4	0.96	2.101 ± 1.063	1.5	1.02	4.005 ± 3.729
α16	+	3.03	0.97	2.83 ± 0.270	1.66	1.15	2.767 ± 0.298
α20	+?	1.93	0.8	2.154 ± 0.204	1.84	0.86	1.543 ± 0.018
α29	+	1.54	1.08	1.434 ± 0.086	1.88	1.32	1.302 ± 0.2

		Standard serum supplement			Reduced serum supplement		
Cells	Score	Lag (days)	D.T (days)	Saturation density ± S.E. ($x10^5$)	Lag (days)	D.T. (days)	Saturation density ± S.E. ($x10^5$)
α32	+?	2.42	0.79	2.191 ± 0.189	2.13	0.91	1.732 ± 0.163
α46	+?	1.64	0.7	1.647 ± 0.150	1.78	0.85	1.115 ± 0.151
α2	X/+	2.36	1.63	1.208 ± 0.348	2.86	2.04	0.677 ± 0.343
α5	X/+	1.44	0.53	3.541 ± 0.307	1.18	0.71	2.908 ± 0.241
α4	-	0.9	0.62	2.256 ± 0.219	1.45	0.99	2.089 ± 0.219
α36	-?	1.28	0.89	2.268 ± 0.292	1.45	1.01	1.992 ± 0.401
α41	-	1.75	1.06	2.577 ± 0.204	1.27	0.89	2.267 ± 0.6
α55	-	1.43	0.66	3.583 ± 0.153	0.98	0.68	2.751 ± 0.172
Spontaneous Focus							
	+	1.78	1.03	3.393 ± 0.106	2.11	1.1	2.439 ± 0.132
Tumour cells from the spontaneous focus							
	+	1.36	0.65	3.71 ± 0.132	1.34	0.83	2.359 ± 0.122
Untransformed C3H10T½ cells							
		1.34± 0.31	0.67 ± 0.13	3.589 ± 0.053	1.54 ± 0.43	0.79 ± 0.16	3.662 ± 0.073

Appendix 5.4.2. Data on the individual alpha-particle induced foci and tumour cell lines, spontaneous focus and tumour cells, and the untransformed C3H10T½ cells. Table shows the lag time (lag), doubling time (D.T.) and saturation density (± standard error) of the cell lines. Data are combined and presented in appendices 5.4.3. to 5.4.5. Growth parameters were calculated for cells grown in culture medium supplemented with 10% foetal calf serum (standard) and with 5% foetal calf serum (reduced).

	Standard serum supplement			Reduced serum supplement		
Score	Lag ± S.E. (days)	D.T. ± S.E. (days)	Saturation density ± S.E. ($x10^5$)	Lag ± S.E. (days)	D.T. ± S.E. (days)	Saturation density ± S.E. ($x10^5$)
X-ray induced foci						
(+) n=9	1.82 ± 0.51	0.91 ± 0.11	3.83 ± 0.07	2 ± 0.72	1.09 ± 0.25	2.551 ± 0.050
(X/+) n=4	2.11 ± 0.75	1.17 ± 0.39	3.208 ± 0.096	3.12 ± 1.87	1.36 ± 0.78	1.79 ± 0.148
(X) n=2	1.41 ± 0.28	0.65 ± 0.16	3.434 ± 0.086	1.53 ± 0.27	0.76 ± 0.13	2.693 ± 0.086
(-) n=5	2.18 ± 0.93	1.07 ± 0.35	3.293 ± 0.081	3.29 ± 1.88	1.09 ± 0.32	2.428 ± 0.077
Alpha-particle induced foci						
(+) n=11	2.25± 0.88	0.78 ± 0.22	2.36 ± 0.088	1.99 ± 1.01	0.79 ± 0.17	2.114 ± 0.082
(X/+) n=5	1.54 ± 0.35	0.83 ± 0.09	3.202 ± 0.058	1.84 ± 0.36	1.02 ± 0.13	2.404 ± 0.059
(X) n=1	1.35	0.63	2.49 ± 0.094	1.63	0.71	2.09 ± 0.068
(-) n=5	1.45 ± 0.5	0.7 ± 0.18	4.0 ± 0.115	1.41 ± 0.43	0.8 ± 0.2	2.886 ± 0.106
Combined X-ray and alpha-particle data						
(+) n=20	2.03 ± 0.65	0.87± 0.16	3.12 ± 0.052	2.08 ± 0.74	0.94 ± 0.26	2.43 ± 0.038
(X/+) n=9	1.79 ± 0.6	0.98 ± 0.3	3.2 ± 0.054	2.41 ± 1.35	1.17 ± 0.52	2.13 ± 0.073
(X) n=3	1.39 ± 0.2	0.64 ± 0.12	3.12 ± 0.065	1.56 ± 0.2	0.74 ± 0.1	2.49 ± 0.062
(-) n=10	1.82 ± 0.8	0.89 ± 0.33	3.65 ± 0.070	2.35 ± 1.63	0.94 ± 0.3	2.657 ± 0.066

Appendix 5.4.3. Data on the groups of foci (includes tumourigenic and non - tumourigenic foci) induced by alpha-particle and X-rays are presented. Also presented are the combined radiation data. Table shows the lag time (lag), doubling time (D.T.) and saturation density of both the tumourigenic and non - tumourigenic focus cell lines. Growth parameters were calculated for cells grown in culture medium supplemented with 10% foetal calf serum (standard) and with 5% foetal calf serum (reduced). Data are also presented in graphs (a) of 5.4.2. to 5.4.4.

Score	Standard serum supplement			Reduced serum supplement		
	Lag ± S.E. (days)	D.T. ± S.E. (days)	Saturation density ± S.E. ($x10^5$)	Lag ± S.E. (days)	D.T. ± S.E. (days)	Saturation density ± S.E. ($x10^5$)
X-ray induced foci						
(+) n=6	1.86 ± 0.54	0.88 ± 0.06	4.44 ± 0.094	2.17 ± 0.83	1.09 ± 0.3	2.776 ± 0.066
(X/+) n=3	2.22 ± 0.87	1.18 ± 0.47	3.379 ± 0.123	3.43 ± 2.16	1.31 ± 0.95	2.275 ± 0.177
(X) n=1	1.21	0.76	4.695 ± 0.122	1.34	0.85	3.607 ± 0.126
(-) n=4	2.35 ± 0.98	1.14 ± 0.38	3.018 ± 0.090	3.73 ± 1.86	1.17 ± 0.31	2.093 ± 0.066
Alpha-particle induced foci						
(+) n=6	2.29± 0.81	0.82 ± 0.22	2.066 ± 0.086	2.12 ± 0.99	0.83 ± 0.19	2.132 ± 0.077
(X/+) n=2	1.59 ± 0.02	0.93 ± 0.04	2.767 ± 0.104	2.09 ± 0.15	1.15 ± 0.01	2.217 ± 0.115
(-) n=4	1.47 ± 0.57	0.65 ± 0.15	3.656 ± 0.093	1.32 ± 0.45	0.71 ± 0.07	2.769 ± 0.950
Combined X-ray and alpha-particle data						
(+) n=12	2.05 ± 0.72	0.83± 0.16	3.401 ± 0.064	2.08 ± 0.89	0.95 ± 0.28	2.44 ± 0.052
(X/+) n=5	1.79 ± 0.6	0.98 ± 0.3	3.204 ± 0.054	1.25 ± 0.68	2.89 ± 1.7	2.03 ± 0.116
(X) n=1	1.21	0.76	4.695 ± 0.122	1.34	0.85	3.61 ± 0.126
(-) n=8	1.91 ± 0.88	0.89 ± 0.37	3.34 ± 0.065	2.52 ± 1.8	0.94 ± 0.32	2.431 ± 0.058

Appendix 5.4.4. Data on the groups of foci (tumourigenic foci only) induced by alpha-particle and X-rays are presented. Also presented are the combined radiation data. Table shows the lag time (lag), doubling time (D.T.) and saturation density of the tumourigenic focus cell lines only. Growth parameters were calculated for cells grown in culture medium supplemented with 10% foetal calf serum (standard) and with 5% foetal calf serum (reduced). Data are also presented in graphs (b) of 5.4.2. to 5.4.4.

	Standard serum supplement			Reduced serum supplement		
Score	Lag ± S.E. (days)	D.T. ± S.E. (days)	Saturation density ± S.E. ($x10^5$)	Lag ± S.E. (days)	D.T. ± S.E. (days)	Saturation density ± S.E. ($x10^5$)
Tumour cells from X-ray induced foci						
(+) n=6	2.29 ± 0.64	0.62 ± 0.23	2.43 ± 0.112	2.18 ± 0.39	0.81 ± 0.28	1.740 ± 0.079
(X/+) n=3	1.92 ± 0.85	0.68 ± 0.14	3.033 ± 0.154	1.95 ± 0.84	0.92 ± 0.27	2.47 ± 0.123
(X) n=1	2.46	1.07	2.171 ± 0.118	2.2	0.58	2.836 ± 0.117
(-) n=4	2.02 ± 0.16	0.6 ± 0.14	2.783 ± 0.123	2.19 ± 0.7	0.71 ± 0.14	1.803 ± 0.107
Tumour cells from alpha-particle induced foci						
(+) n=6	1.99 ± 0.62	0.88 ± 0.14	2.06 ± 0.191	1.8 ± 0.21	1.02 ± 0.19	2.077 ± 0.063
(X/+) n=2	1.9 ± 0.65	1.08 ± 0.78	2.375 ± 0.232	2.02 ± 1.19	1.38 ± 0.94	1.793 ± 0.21
(-) n=4	1.34 ± 0.35	0.81± 0.21	2.67 ± 0.111	1.29 ± 0.22	0.89 ± 0.15	2.274 ± 0.193
Combined tumour cells data from X-ray and alpha-particle induced foci						
(+) n=12	2.07 ± 0.64	0.76± 0.22	2.29 ± 0.105	1.95 ± 0.37	0.92 ± 0.24	1.92 ± 0.292
(X/+) n=5	1.91 ± 0.69	0.84 ± 0.46	2.77 ± 0.131	1.98 ± 0.84	1.1 ± 0.57	2.2 ± 0.112
(X) n=1	2.46	1.07	2.17 ± 0.118	2.2	0.58	2.84 ± 0.117
(-) n=8	1.68 ± 0.44	0.70 ± 0.2	2.73 ± 0.083	1.74 ± 0.68	0.8 ± 0.17	2.039 ± 0.111

Appendix 5.4.5. Data on the groups of tumour cells from the different categories of foci (+), (X/+), (X), and (-) induced by alpha-particle and X-rays are presented. Also presented are the combined radiation data. Table shows the lag time (lag), doubling time (D.T.) and saturation density of the cell lines. Growth parameters were calculated for cells grown in culture medium supplemented with 10% foetal calf serum (standard) and with 5% foetal calf serum (reduced). Data are also presented in graphs (b) of 5.4.2. to 5.4.4.

	Ratio of standard: reduced serum supplement			Ratio of standard: reduced serum supplement		
Score	Lag ± S.E.	D.T. ± S.E.	Saturation density ± S.E.	Lag ± S.E.	D.T. ± S.E.	Saturation density ± S.E.
	X-ray induced foci			Tumour cells from X-ray induced foci		
(+)	0.96 ± 0.27	0.86 ± 0.15	1.58 ± 0.53	1.02 ± 0.18	0.79 ± 0.12	1.61 ± 0.33
(X/+)	0.74 ± 0.16	0.96 ± 0.29	1.74 ± 0.65	0.98 ± 0.07	0.78 ± 0.23	1.23 ± 0.17
(X)	0.92 ± 0.02	0.85 ± 0.06	1.26 ± 0.06	0.89	0.54	1.31
(-)	0.72 ± 0.15	0.99 ± 0.09	1.46 ± 0.31	1.0 ± 0.30	0.84 ± 0.09	1.71 ± 0.61
	Alpha-particle induced foci			Tumour cells from alpha-particle induced foci		
(+)	1.1 ± 0.4	1.03 ± 0.17	1.2 ± 0.22	1.11 ± 0.37	0.87 ± 0.05	1.13 ± 0.34
(X/+)	0.85 ± 0.15	0.81 ± 0.03	1.37 ± 0.26	1.02 ± 0.28	0.77 ± 0.04	1.5 ± 0.4
(X)	0.83	0.89	1.19			
(-)	1.04 ± 0.21	0.88 ± 0.15	1.38 ± 0.23	1.09 ± 0.4	0.92 ± 0.23	1.16 ± 0.1

Appendix 5.4.6. Data on the ratio of standard : reduced serum conditions of lag times, doubing times and saturation densities for the foci and tumour cells. Growth parameters were calculated for cells grown in culture medium supplemented with 10% foetal calf serum (standard) and with 5% foetal calf serum (reduced). The ratios were calculated for individual cell lines and these ratios combined to produce a ratio for the different categories of foci and tumour cell lines.

Section 5.5.

Results of Cytogenetics Studies

List of tables and figures

There is strong circumstantial evidence that damage to DNA which carries the genetic information in chromosomes in the cell nucleus is the main target for the biological effects of ionising radiation, including cell killing, mutation and carcinogenesis (for example, UNSCEAR 1993, Hall 1994). DNA damage may take the form of base damage, single- or double- strand breaks and multiple damage sites in the DNA (Tubiana *et al.* 1990, Ward 1995). Misrepair of this damage leads to aberrations in the chromosomes which in turn may lead to activation of proto-oncogenes or inactivation of tumour suppressor genes. Untransformed C3H10T½ cells are hypertetraploid with a small proportion of cells in the octaploid range (Reznikoff *et al.* 1973). This section presents data on the variation of chromosome number in the different categories of transformed focus cells ((+), (X/+), (X), (-)) and their corresponding tumour cells, induced by X-rays or alpha-particles. During the course of this study a number of metacentric chromosomes (Robertsonian chromosomes) were observed in the metaphases. Mouse chromosomes are acrocentric, and appear as V - shaped in metaphase spreads with the centromere at the base of the V, while metacentric chromosomes are X-shaped with the centromere located in the middle of the X (see figure 5.5.1). Robertsonian fusions were first described by W.R.B Robertson in 1916 who concluded from observations that a metacentric chromosome in one species may correspond to two acrocentrics in another, that during evolution metacentrics may arise by fusion of acrocentrics (Robertson 1916). Whole chromosome arm fusions are called Robertsonian translocations in his honour. They are the most common structural chromosome abnormality in humans, one in a thousand in the general population (Wolff *et al.* 1992), can occur spontaneously and are not thought to be specifically induced by ionising radiation.

Data are presented for changes in the tetraploid state of the C3H10T½ cells and for the presence or absence of Robertsonian translocations in the various C3H10T½ cell lines examined. The mean chromosome numbers of the different foci and their corresponding tumour cells were examined for significant differences using the student *t* - test at the five percent confidence level.

Table 5.5.1 shows the mean and modal chromosome number of the individual cell lines beginning with the foci in the order of focus category (+), (X/+), (X), (-),

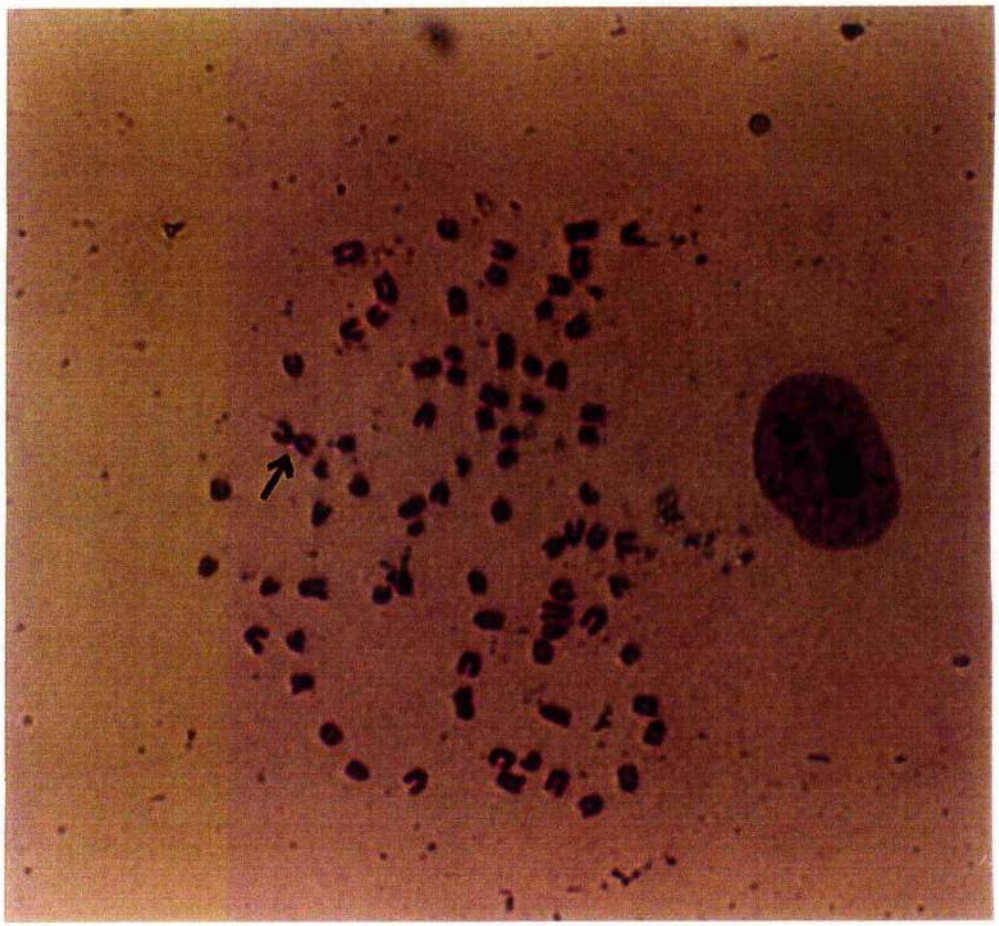

Figure 5.5.1. Photograph of the chromosomes of an X-ray induced (X/+) focus (X4). Most of the chromosomes are acrocentric however one metacentric (Robertsonian) chromosome is also present (arrow). The line under the photograph represent 0.05 millimetres as determined using photographs of a 1mm Objective micrometer (Nikon).

and followed by the corresponding tumour cells. The mean chromosome number represents the average number over all the metaphases examined with the standard deviation indicating the spread of the numbers while the modal number indicates the number of chromosomes in the majority of the metaphases examined. At least a hundred metaphases were examined for each cell line. It is apparent from the table that the majority of the cell lines (focus / tumour) have mean and modal chromosome numbers which are lower than that of the untransformed cells. Cell lines which have higher modal chromosomal numbers are one focus cell line (X14) and three tumour cell lines (X4, α9, STR). Higher mean chromosome numbers than that of the untransformed cells are shown by ten focus cell lines and six tumour cell lines. Also presented in table 5.5.1 are the percentage Robertsonian chromosomes observed in each cell line.

These data are presented in the following figures where the cell lines have been grouped according to the focus categories and radiation treatment. Figure 5.5.2 presents the distribution of chromosome number in untransformed C3H10T½ cells and spontaneous cell lines. In each set of figures 5.5.3, 5.5.4, 5.5.5 the first figure (a) illustrates the distribution of chromosome number in metaphases of radiation induced foci (combined data of X-rays and alpha-particles), while the second figure (b) shows the data for the X-ray induced foci and the last figure (c) in each set shows the data for the alpha-particle induced foci. The first set of figures (5.5.3) illustrate the distribution of chromosome number in metaphases of tumourigenic and non - tumourigenic foci (combined data) while the second set of figures (5.5.4) illustrate the distribution of chromosome number in metaphases of tumourigenic foci only. Figures 5.5.5 (a,b,c) show the distribution of chromosome number in metaphases of the tumour cells.

Figures 5.5.6 (a, b, c, d) present the distribution of chromosome number in metaphases of (a) (+), (b) (X/+), (c) (X) and (d) (-) focus and tumour cells. Figure 5.5.7 presents the percentage metaphases of the different focus and tumour cell categories which displayed Robertsonian chromosomes.

Table 5.5.1. Mean and modal chromosome number per cell with percentage Robertsonian chromosomes.

		Focus cells			Tumour cells		
Cells	Score	Mean ± S.D.	Modal	Percentage Robertsonian translocations	Mean ± S.D.	Modal	Percentage Robertsonian translocations
X1	(+)	68 ± 10	68	6.9	63 ± 10	58/63	0
X3	(+)	74 ± 9	75	6	72 ± 11	68	25
X5	(+)	75 ± 18	70	7	71 ± 4	72	32
X7	(+)	75 ± 17	70	1	73 ± 8	70	33
X9	(+)	73 ± 5	73	3	75 ± 19	70	6
X10	(+)	71 ± 19	67	10	68 ± 4	70	13
X19	(+)	67 ± 15	65/61	4	71 ± 21	66	16
X27	(+)	72 ± 5	70	0.9	74 ± 13	72	4
Mean X-ray (+) data		72 ± 13		4.9 ± 3	71 ± 13		16.1 ± 12
α1	(+)	89 ± 29	71	1.8			
α10	(+)	72 ± 8	74	0	65 ± 9	66/67	0
α12	(+)	75 ± 19	72	15			
α18	(+)	73 ± 9	74	2.9			

		Focus cells			Tumour cells		
Cells	Score	Mean ± S.D.	Modal	Percentage Robertsonian translocations	Mean ± S.D.	Modal	Percentage Robertsonian translocations
α19	(+)	70 ± 7	70	2	73 ± 5	74	2
Mean alpha-particle (+) data		76 ± 17		4.3 ± 4.3	73 ± 5	74	0
STR	(+)	90 ± 28	73	1.4	107 ± 18	98/100	4
X4	(X/+)	75 ± 12	75	51	98 ± 26	120	2
X12	(X/+)	69 ± 6	71	18			
Mean X-ray (X/+) data		72 ± 9		35 ± 17	98 ± 26	120	2
α2	(X/+)	69 ± 9	71/73	7	68 ± 14	65	0
α5	(X/+)	73 ± 18	60/65	1	68 ± 7	65	3
α13	(X/+)	67 ± 9	68	3			
α22	(X/+)	65 ± 8	67	0			
Mean alpha-particle (X/+) data		69 ± 12		2.8 ± 2.6	68 ± 11		1.5 ± 1.5

		Focus cells			Tumour cells		
Cells	Score	Mean ± S.D.	Modal	Percentage Robertsonian translocations	Mean ± S.D.	Modal	Percentage Robertsonian translocations
X2	(X)	72 ± 11	72	1	75 ± 24	66	0
X11	(X)	75 ± 6	73	0			
Mean X-ray (X) data		74 ± 9		0.5 ± 0.5	75 ± 24	66	0
α51	(X)	75 ± 5	75	0			
X6	(-)	70 ± 12	69	0			
X14	(-)	99 ± 16	107	2	80 ± 15	75	2.6
X18	(-)	65 ± 10	65	0	65 ± 16	60	3
Mean X-ray (-) data		78 ± 13		0.7 ± 0.7	73 ± 16		2.8 ± 2.8
α4	(-)	72 ± 9	70	0	74 ± 11	74	6
α9	(-)	67 ± 6	63	0	114 ± 12	118	2.5
α24	(-)	79 ± 23	70/72/73	9			
α41	(-)	70 ± 6	70	15	71 ± 11	68	3

		Focus cells			Tumour cells		
Cells	Score	Mean ± S.D.	Modal	Percentage Robertsonian translocations	Mean ± S.D.	Modal	Percentage Robertsonian translocations
Mean alpha-particle (-) data		72 ± 13		6 ± 6	86 ± 11		3.8 ± 1.5
Untransformed		74 ± 4	77	6.3			

Table shows the mean (± standard deviation (S.D.)) of the chromosome number as well as the modal chromosome number of the C3H10T½ cell lines examined. Also shown is the percentage metaphases observed containing a Robertsonian chromosome. The cell lines are grouped according to focus category in the order (+), (X/+), (X) and (-) beginning with the transformed cell lines followed by the untransformed cells. STR refers to the spontaneous focus / tumour cells. Labels beginning with X refer to the X-ray induced focus / tumour cells while those beginning with α indicate the alpha-particle induced focus / tumour cells.

Untransformed and spontaneously transformed cells

Figure 5.5.2 presents the distribution of chromosome number in the metaphases of untransformed C3H10T½ cells, spontaneous focus and tumour cells. The tight distribution of numbers for the untransformed cells is contrasted with a greater spread of chromosome numbers for both the focus and tumour cells of the spontaneous focus. The mean chromosome number is significantly different ($p < 0.05$) for the untransformed cells compared to both the focus and tumour cells of the spontaneous focus examined. While the modal chromosome number of the spontaneous focus (65-69) is lower than that of the untransformed cells (75-79) the mode of the tumour cells from the spontaneous focus is higher (100-104). Mean chromosome numbers of both focus and tumour cells are higher than that of the untransformed cells (see table 5.5.1).

Combined data of distribution of chromosome numbers of tumourigenic and non - tumourigenic foci

Figure 5.5.3 (a) illustrates the distribution of the chromosome numbers of the radiation induced foci. These are the combined data of the tumourigenic and non - tumourigenic foci induced by X-rays and alpha-particles. Most of the metaphases examined have chromosome numbers in the range 55 to 84 chromosomes per metaphase although the complete distribution is quite wide. The modal chromosome number of the (+) and (X) foci is slightly higher than that of the (X/+) and (-) foci although the only significant differences in the means ($p < 0.05$) is between the (X/+) foci and all other categories. The distribution of the chromosome numbers for all the categories of foci is much greater than that of the untransformed cells (see figure 5.5.2).

Figure 5.5.3 (b) shows the distribution of chromosome number in the metaphases of the X-ray induced foci (combined data of tumourigenic and non - tumourigenic foci). The modal chromosome numbers of the (+), (X/+) and (X) foci are in the same range while that of the (-) foci is lower. Similarly the mean chromosome number of the (-) foci is significantly different ($p < 0.05$) to all other categories while no difference is found between the means of the (+), (X/+) and (X) foci. The distribution of the chromosome numbers of the (-) foci appears different to

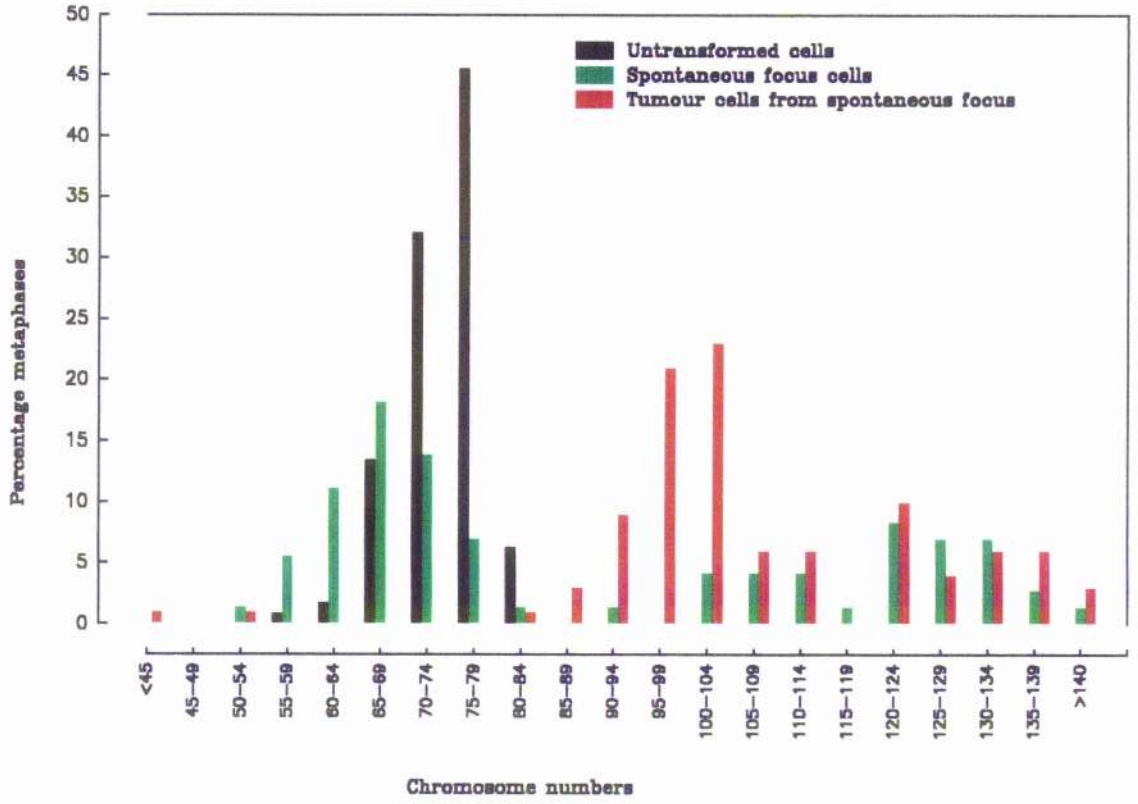

Figure 5.5.2.Distribution of chromosome number in metaphases of untransformed C3H10T½ cells, spontaneous focus cells and tumour cells developed from the spontaneous focus.

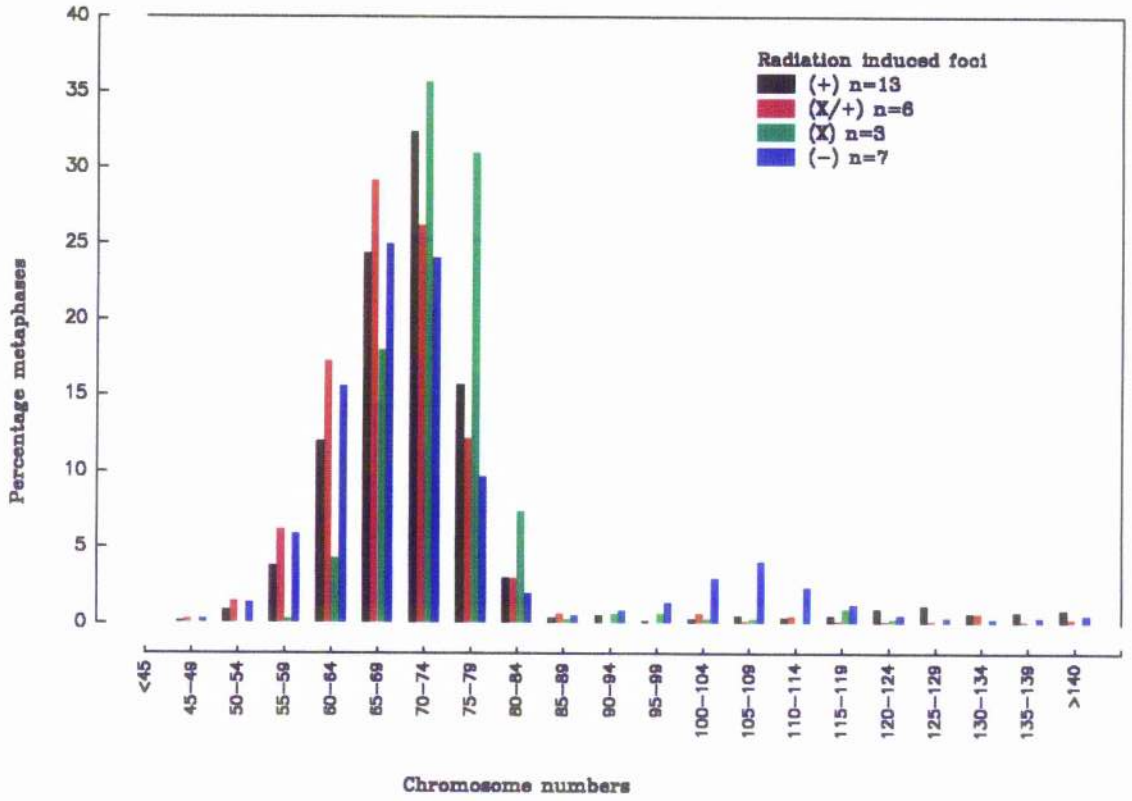

Figure 5.5.3 (a). Distribution of chromosome number in metaphases of radiation induced foci, combined data of tumourigenic and non tumourigenic foci induced by X-rays and alpha - particles (n = number of cell lines examined).

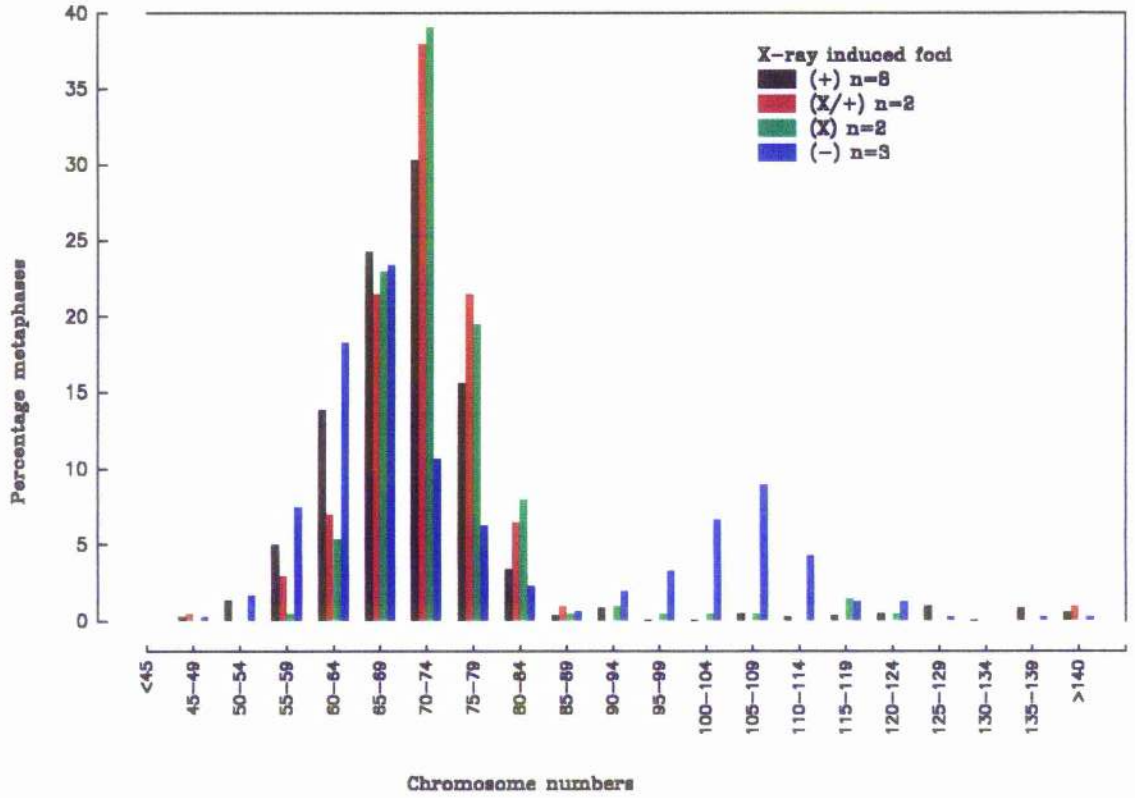

Figure 5.5.3 (b). Distribution of chromosome number in metaphases of X-ray induced foci, combined data of tumourigenic and non tumourigenic foci (n = number of cell lines examined).

that of the other categories especially in the range of 90 to 124 chromosomes per metaphase.

Figure 5.5.3 (c) shows the distribution of chromosome number in the metaphases of the alpha-particle induced foci (combined data of tumourigenic and non - tumourigenic foci). The modal chromosome number of the (X) focus is higher than all other categories with a higher percentage of metaphases containing the modal number of chromosomes, followed by that of the (+) and (-) foci in the range 70 to 74 chromosomes per metaphase while the modal chromosomal number of the (X/+) foci is in the lowest range of all the focus categories. The mean chromosome number of the (X) focus is significantly different ($p < 0.05$) to that of other categories except the (+) foci. Significant differences ($p < 0.05$) also exist between the mean chromosome numbers of the (+) foci, (X/+) and (-) foci. From the graph one can also observe the distribution of the chromosome number of the (X) focus is smaller than that of the other categories although this is based on only one focus.

Distribution of chromosome numbers of tumourigenic foci

Figure 5.5.4 (a) shows the distribution of chromosome number in the metaphases of the radiation induced tumourigenic foci. These are the combined data of X-ray and alpha-particle induced tumourigenic foci. The modal chromosome numbers of (+), (X/+) and (X) foci are all in the range 70 to 74 chromosomes per metaphase while that of the (-) foci is slightly lower. No great differences exist between the different focus categories in the mean chromosome number ($p < 0.05$). The distribution of chromosome numbers for all the focus categories is wide with most of the metaphases showing numbers in the 55 to 84 chromosomes per metaphase range, although the (-) foci show another peak in chromosome number in the 105 to 109 chromosomes per metaphase range.

Figure 5.5.4 (b) shows the distribution of chromosome number in the metaphases of the X-ray induced tumourigenic foci. The modal chromosome number of all focus categories (except (-)) is in the range 70 to 74 chromosomes per metaphase, while that of the (-) foci is in the 60 to 69 chromosomes per metaphase range. The mean chromosome number of the (-) foci is significantly different ($p <$

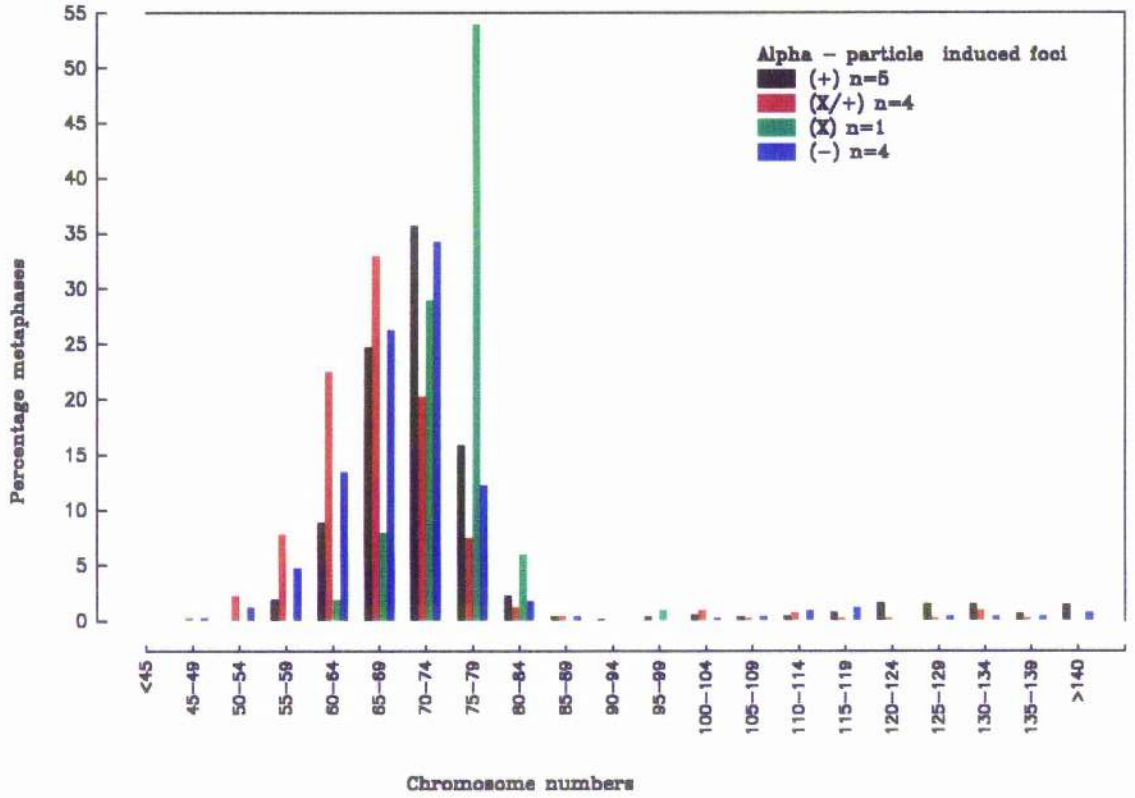

Figure 5.5.3 (c). Distribution of chromosome number in metaphases of alpha -particle induced foci, combined data of tumourigenic and non tumourigenic foci (n = number of cell lines examined).

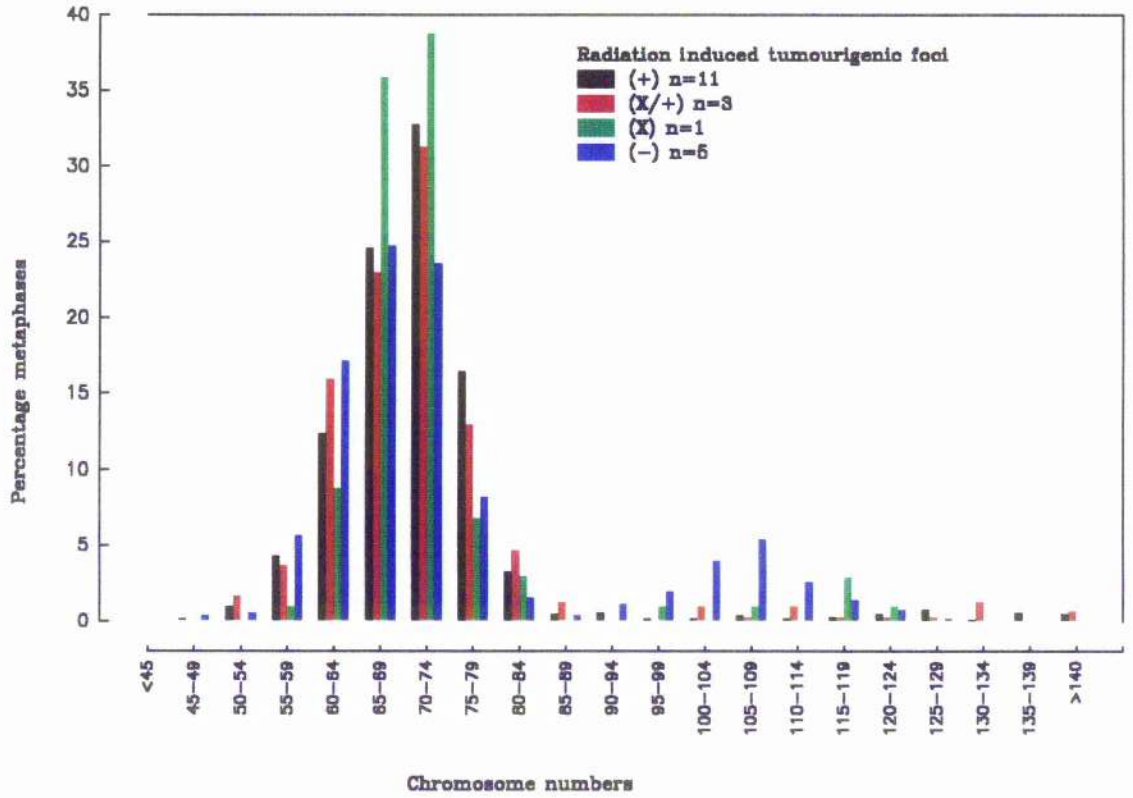

Figure 5.5.4 (a). Distribution of chromosome number in metaphases of radiation induced foci, combined data of X-ray and alpha - particle induced tumourigenic foci (n = number of cell lines examined).

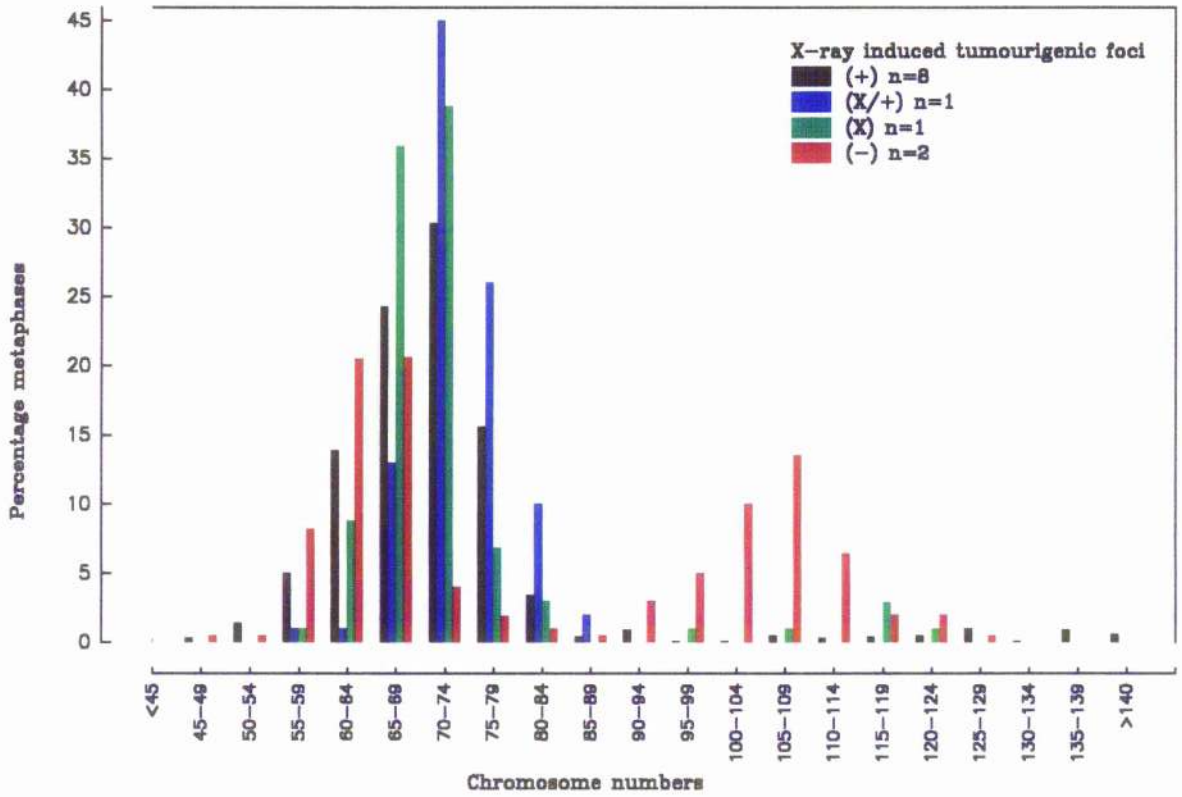

Figure 5.5.4 (b). Distribution of chromosome number in metaphases of X-ray induced tumourigenic foci (n = number of cell lines examined).

0.05) to that of all other categories. As noted in the previous figure the distribution of chromosome numbers for all the focus categories is broad with most of the metaphases showing 55 to 84 chromosomes while the (-) foci show another peak in chromosome number in the 105 to 109 chromosomes per metaphase range.

Figure 5.5.4 (c) shows the distribution of chromosome number in the metaphases of the alpha-particle induced tumourigenic foci. No tumourigenic (X) foci were available for examination, thus the comparison is between (+), (X/+) and (-) foci. The distribution of chromosome numbers is not as broad as in the previous figures. The modal chromosome numbers of the (+) and (-) foci are similar and higher than that of the (X/+) foci, while a significant difference ($p < 0.05$) exists in the mean chromosome numbers of the (+) and (-) foci.

Distribution of chromosome numbers of tumour cells

Figure 5.5.5 (a) shows the distribution of chromosome numbers in the metaphases of the tumour cells produced by the radiation induced foci. These are the combined data of tumour cells from X-ray and alpha-particle induced foci. A broad distribution of chromosome numbers is observed with a greater percentage of metaphases showing higher chromosome numbers than observed for the foci in previous figures. The distribution is particularly broad for the tumour cells from the (-) foci, and two peaks in chromosome numbers are observed for the tumour cells from the (X) focus (65 to 69 and 115 to 119 chromosomes per metaphase). The modal chromosome number of the (+) cells is higher than all other categories. These other categories have modal chromosomal numbers which all appear in the same range. The mean chromosomal number of the (+) foci is significantly different ($p < 0.05$) to that of the other categories with no great differences between the other categories.

Figure 5.5.5 (b) shows the distribution of chromosome number in the metaphases of the tumour cells produced by the X-ray induced foci. The modal chromosome number is highest for the (X/+) cells (120 to 124 chromosomes per metaphase), followed by the (+) cells (70 to 74 chromosomes per metaphase), then the (X) cells (65 to 69 chromosomes per metaphase) and lastly the (-) tumour cells (60 to 64 chromosomes per metaphase). Mean chromosome numbers are significantly

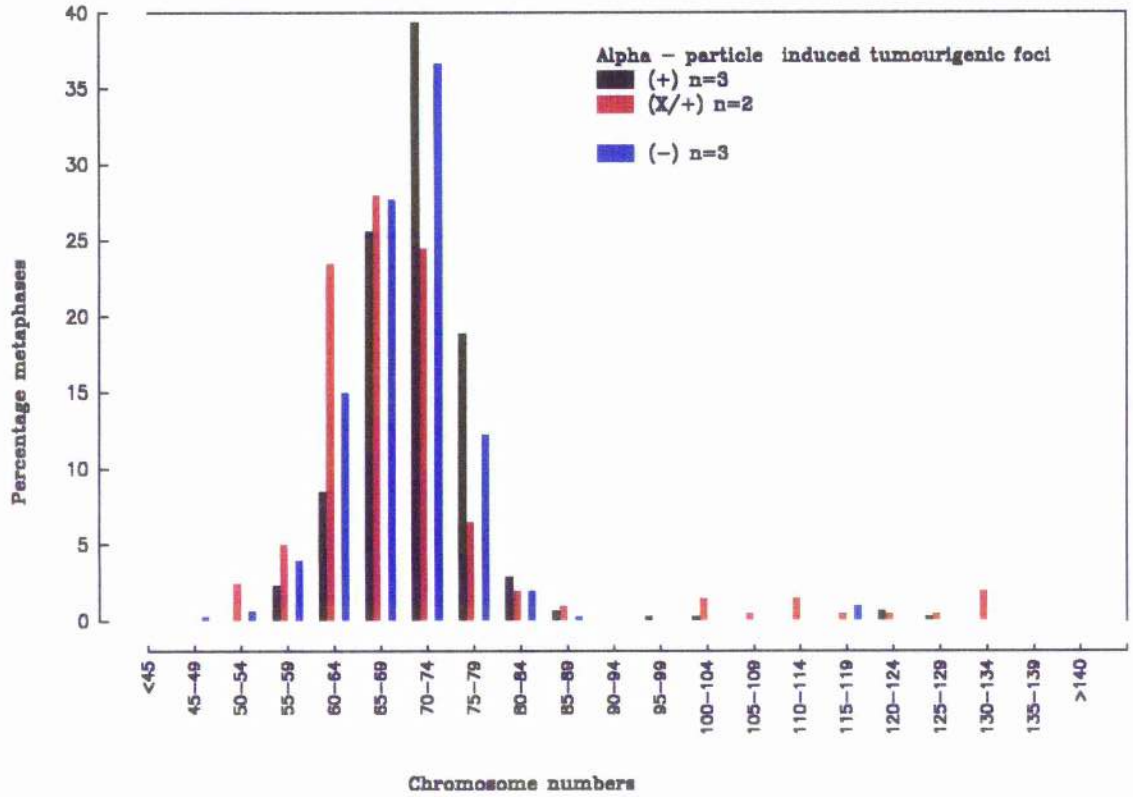

Figure 5.5.4 (c). Distribution of chromosome number in metaphases of alpha - particle induced tumourigenic foci (n = number of cell lines examined).

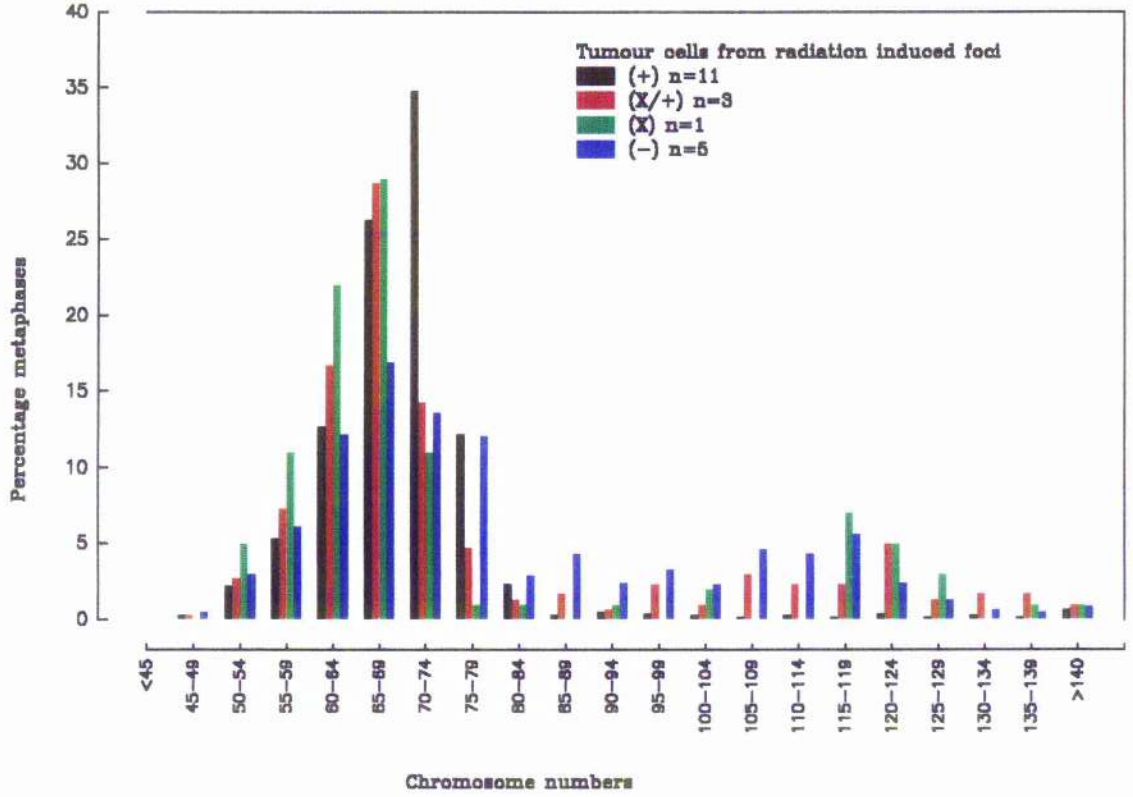

Figure 5.5.5 (a). Distribution of chromosome number in metaphases of tumour cells from radiation induced foci, combined data of foci induced by X-rays and alpha - particles (n = number of cell lines examined).

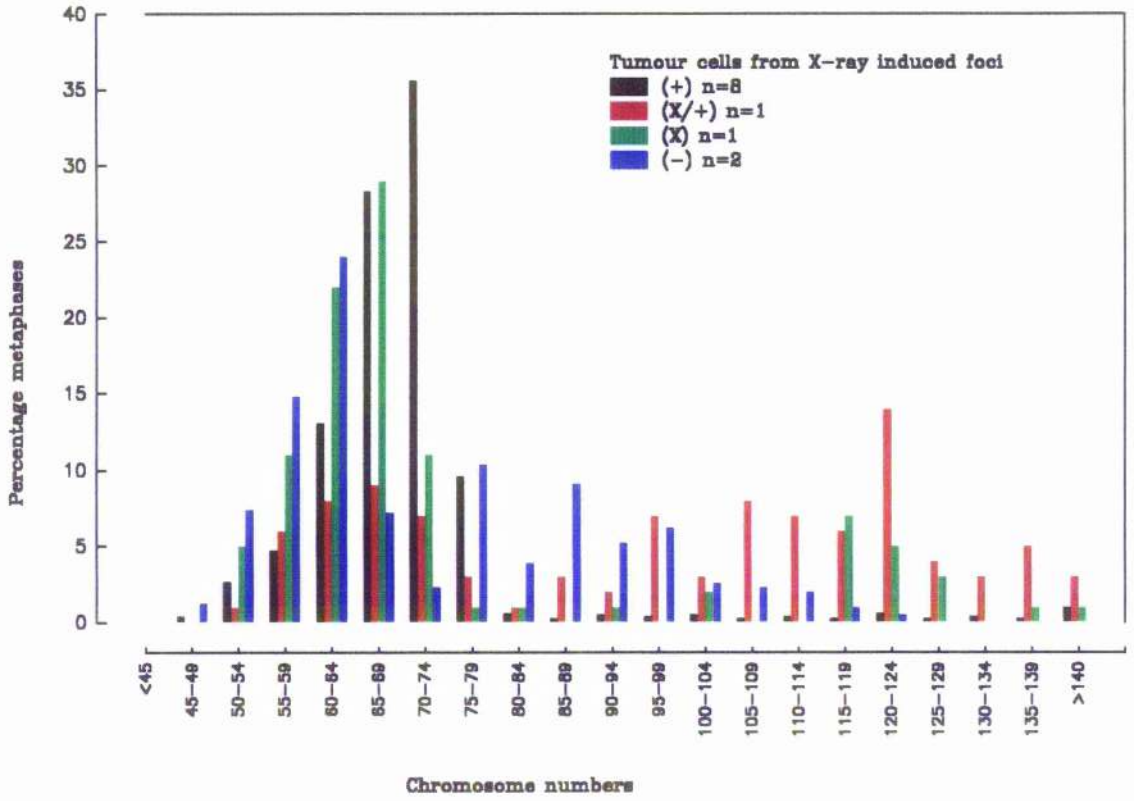

Figure 5.5.5 (b). Distribution of chromosome number in metaphases of tumour cells from X-ray induced foci (n = number of cell lines examined).

different ($p < 0.05$) between each of the categories of tumour cells compared with any other category. The distribution patterns of the chromosome numbers vary between categories of tumour cells with the (+) cells having the most well defined 'bell shape' with a sharp decrease in the percentage of metaphases with greater than 79 chromosomes, while the (-) tumour cells show a much more gradual decline in the percentage metaphases with the higher chromosome numbers. Meanwhile the (X/+) and (X) tumour cells show distributions with two peaks, the first in the range of 60 to 69 chromosomes per metaphase and the second in the range of 120 to 124 chromosomes per metaphase.

Figure 5.5.5 (c) shows the distribution of chromosome number in the metaphases of the tumour cells produced by the alpha-particle induced foci. Chromosome numbers of tumour cells from the (+), (X/+) and (-) foci are compared. The (X/+) and (-) tumour cells have modal chromosome numbers of 65 to 69 chromosomes per metaphase which is below that of the (+) cells. The means of each category are significantly different ($p < 0.05$) between each of the categories of tumour cells compared with any other category. As observed for the tumour cells from the X-ray induced foci the distribution pattern of the (+) cells shows one peak in 70 to 74 chromosomes per metaphase range. The (-) cells have a distribution pattern with two peaks, in the ranges 65 to 74 and 110 to 124 chromosomes per metaphase. The distribution pattern of the (X/+) cells is similar to that of the (+) tumour cells although the peak is in a lower range of chromosome numbers.

Comparison of foci and tumour cells

Figure 5.5.6 (a) shows the distribution of chromosome number in the metaphases of the (+) foci and tumour cells produced by X-rays and alpha-particles. The striking feature in this figure is the narrow range of chromosome numbers compared to previous figures with few metaphases showing greater than 84 chromosome per metaphase. The modal number of chromosomes is in the range 70 to 74 chromosomes per metaphase for both focus and tumour cells induced by both types of radiation. There are no significant differences ($p < 0.05$) between the foci and the corresponding tumour cells or between the X-ray and alpha-particle treated cells

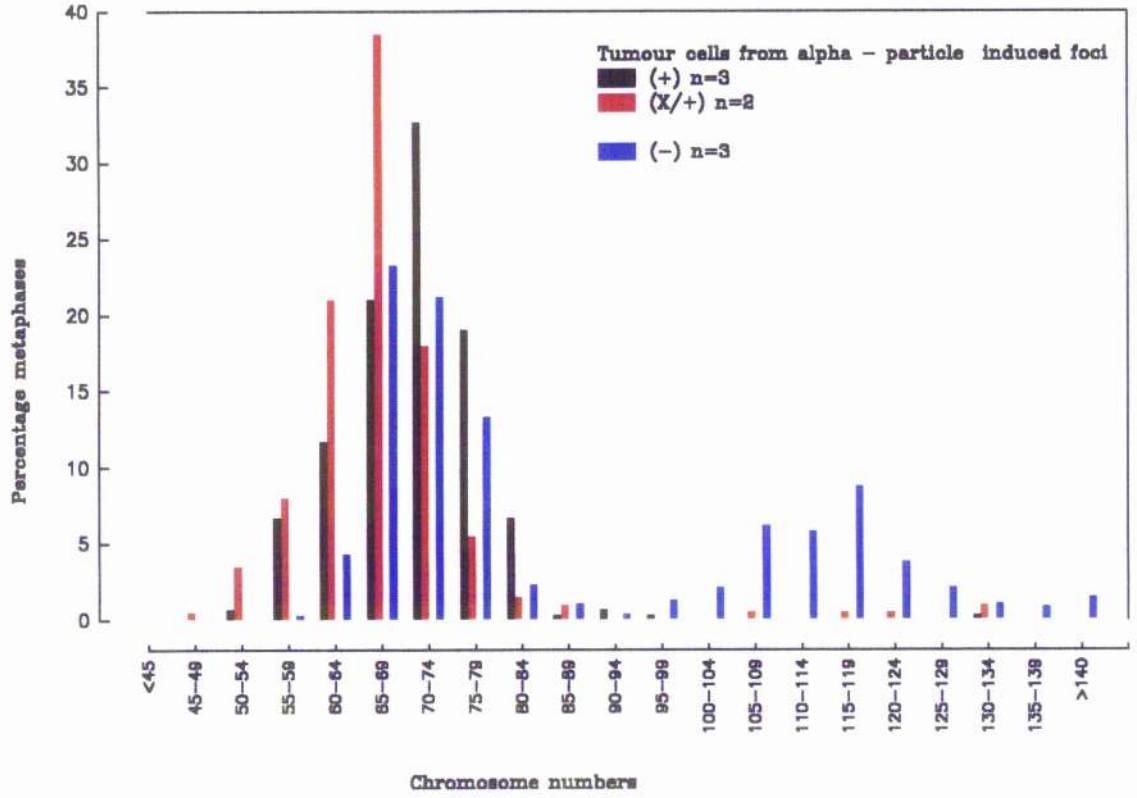

Figure 5.5.5 (c). Distribution of chromosome number in metaphases of tumour cells from alpha - particle induced foci (n = number of cell lines examined).

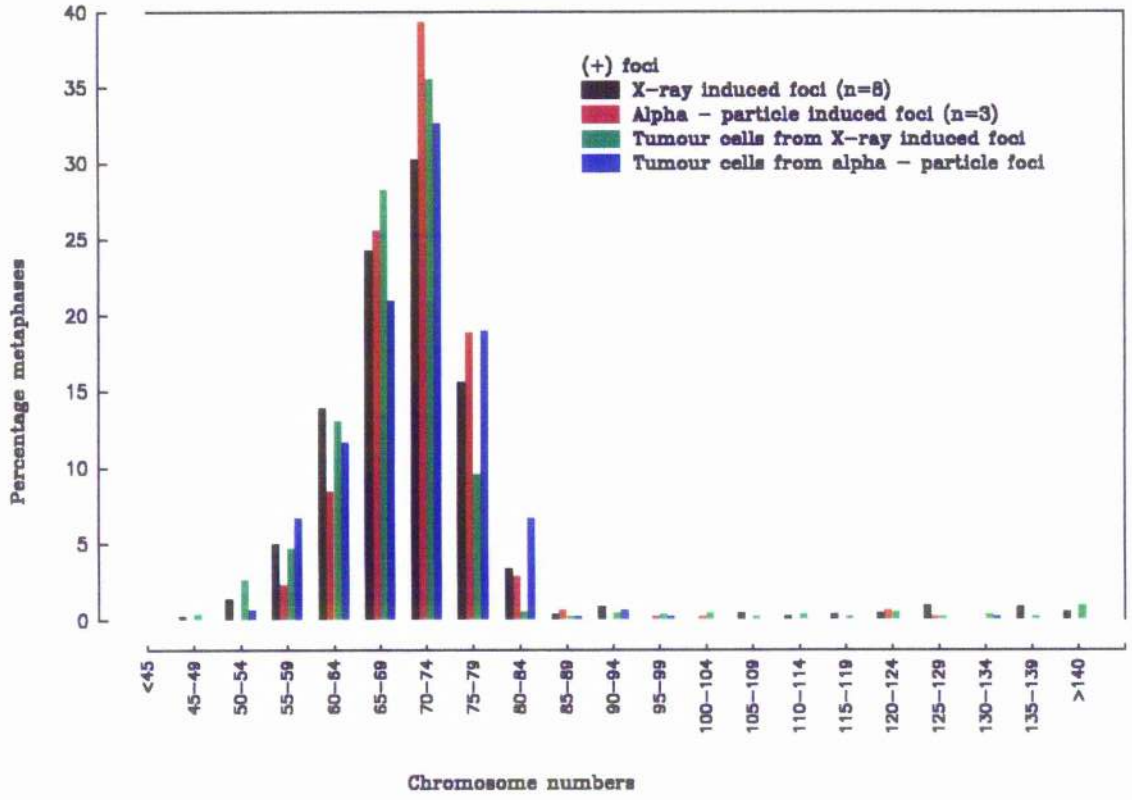

Figure 5.5.6 (a). Distribution of chromosome number in metaphases of tumourigenic (+) foci and corresponding tumour cells (n = number of cell lines examined).

for the mean chromosome number.

Figure 5.5.6 (b) shows the distribution of chromosome number in the metaphases of the (X/+) foci and tumour cells produced by X-rays and alpha-particles. There is a wider spread of chromosome numbers present for the (X/+) cells than was noted in the previous figure for (+) cells, especially for the tumour cells from the X-ray induced (X/+) foci. The modal chromosome number is highest for the tumour cells from the X-ray induced (X/+) foci (120 - 124 chromosomes per metaphase) followed by the X-ray induced foci (70 to 74 chromosomes per metaphase), then the alpha-particle induced foci and corresponding tumour cells (65 to 69 chromosomes per metaphase). The mean chromosome number for the foci and the corresponding tumour cells are significantly different ($p < 0.05$) for both X-rays and alpha-particles. Similarly comparison of X-rays versus alpha-particles shows significant differences ($p < 0.05$) in the mean chromosome number for both the foci and tumour cells.

Figure 5.5.6 (c) shows the distribution of chromosome number in the metaphases of the (X) foci and tumour cells produced by X-rays only. The data show a similar distribution of the chromosome number for both the focus and tumour cells examined with the modal chromosome number slightly higher for the focus than for the tumour cells. The tumour cells show a higher percentage of metaphases with chromosomes in the range of 115 to 129 chromosomes per metaphase while fewer of the metaphases from the focus cells having greater than 84 chromosomes per metaphase. No significant differences ($p < 0.05$) are noted in the mean number of chromosomes of the focus and tumour cells.

Figure 5.5.6 (d) shows the distribution of chromosome number in the metaphases of the (-) foci and tumour cells produced by X-rays and alpha-particles. The distribution pattern of the chromosome numbers of the (-) cells is much broader than noted for any of the other categories. The X-ray induced foci and the tumour cells from the alpha-particle induced foci show two peaks. The peaks for the X-ray induced foci are in the ranges of 60 to 69 and 100 to 109 chromosomes per metaphase while those for tumour cells of the alpha-particle induced foci are in the ranges 65 to 74 and 105 to 119 chromosomes per metaphase. As noted for the (X/+) focus category the mean chromosome number for the foci and the corresponding tumour

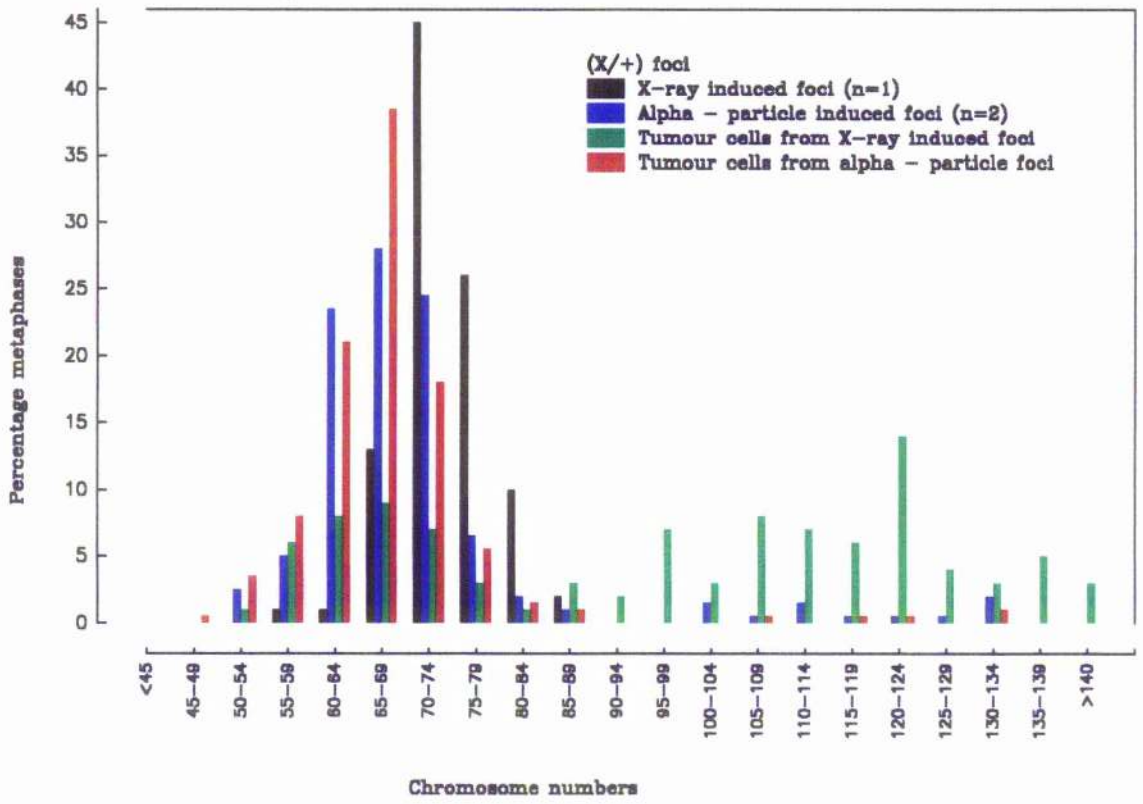

Figure 5.5.6 (b). Distribution of chromosome number in metaphases of tumourigenic (X/+) foci and corresponding tumour cells (n = number of cell lines examined).

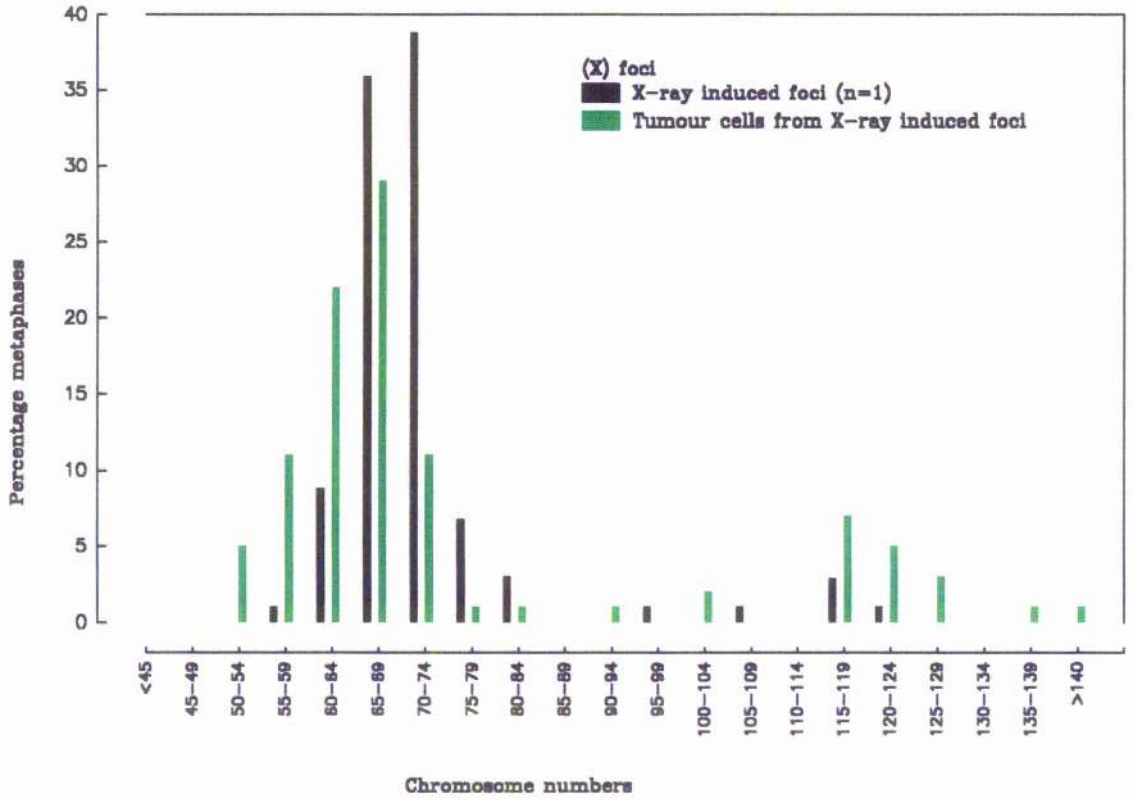

Figure 5.5.6 (c). Distribution of chromosome number in metaphases of tumourigenic (X) foci and corresponding tumour cells (n = number of cell lines examined).

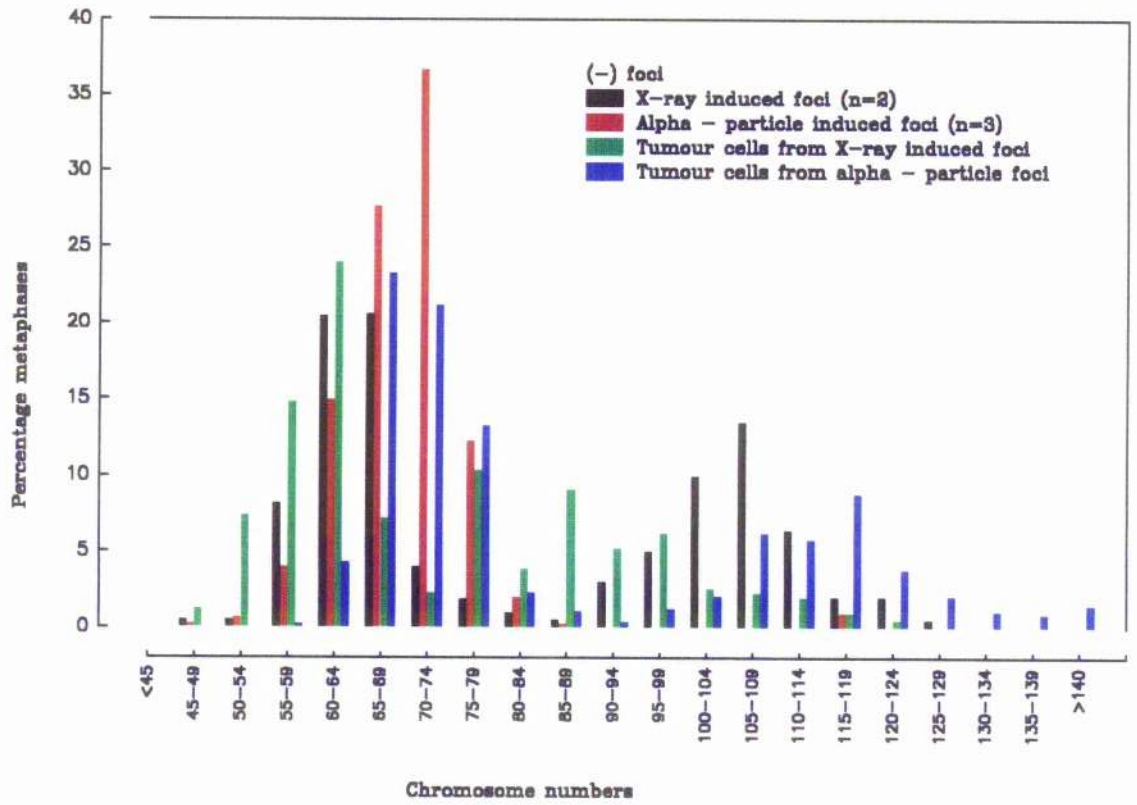

Figure 5.5.6 (d). Distribution of chromosome number in metaphases of tumourigenic (-) foci and corresponding tumour cells (n = number of cell lines examined).

cells are significantly different ($p < 0.05$) for both X-rays and alpha-particles and similarly comparison of X-rays versus alpha-particles shows significant differences ($p < 0.05$) in the mean chromosome number for both the foci and tumour cells examined.

Robertsonian chromosomes

Figure 5.5.7 presents the percentage metaphases with Robertsonian chromosomes. A background level of six percent in the metaphases of the untransformed cells is observed. Few of the focus or tumour cell categories show levels of Robertsonian chromosomes greater than the background level. The tumourigenic and non - tumourigenic X-ray induced (X/+) foci and the tumour cells from the X-ray induced (+) foci are the only cell lines with Robertsonian chromosome levels higher than the background level. Examination of the different focus categories shows that for the (+) foci, the highest level of Robertsonian chromosomes is found in non - tumourigenic alpha-particle induced foci, followed by the tumourigenic X-ray induced foci, leaving the tumourigenic alpha-particle induced foci and the spontaneous focus with the lowest levels of Robertsonian chromosomes. In the corresponding tumour cells from the (+) foci, decreasing numbers of Robertsonian chromosomes are noted in the order of X-rays, spontaneous and lastly alpha-particle treated cells. The X-ray induced (X/+) foci (tumourigenic and non - tumourigenic foci) display a higher percentage of metaphases with Robertsonian chromosomes than the corresponding alpha-particle induced foci, with no differences observed between the tumour cells. The (X) foci and tumour cells show few metaphases with Robertsonian chromosomes. Alpha-particle induced (-) foci show higher levels of Robertsonian chromosomes than the equivalent X-ray induced foci and no differences between tumour cells are observed. Comparison of the foci with their corresponding tumour cells shows higher levels of Robertsonian chromosomes in the tumour cells from the spontaneous focus and X-ray induced (+) foci, and lower levels in the tumour cells of the (X/+) foci (X-ray and alpha-particle induced foci) compared to the foci. No great differences in the levels of Robertsonian chromosomes are noted between the foci and tumour cells of the alpha-particle induced (+) or (-) cells or the X-ray induced (X) or (-) cells.

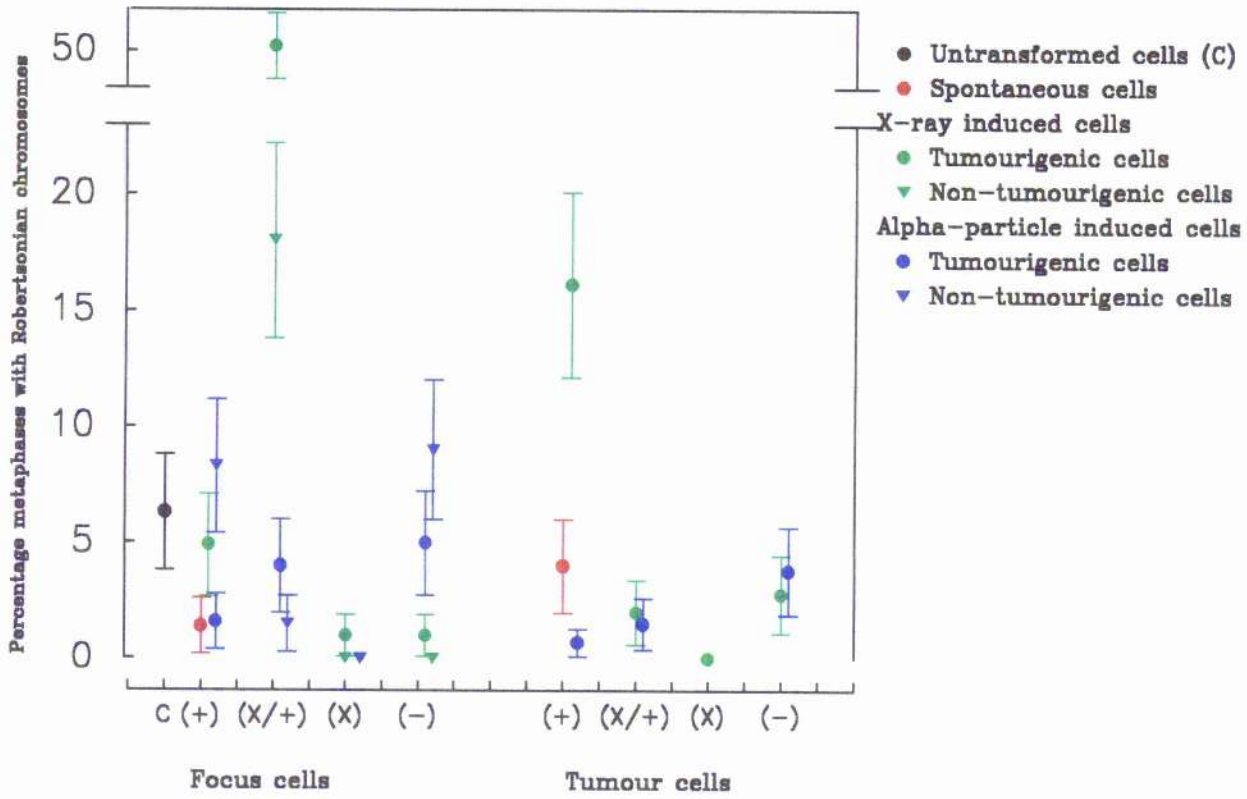

Figure 5.5.7. Percentage metaphases with Robertsonian chromosomes of untransformed C3H10T½ cells, tumour cells and tumourigenic and non-tumourigenic foci induced by X-rays and alpha - particles.

Discussion

The most notable differences in chromosome numbers between the transformed C3H10T½ cell lines examined and the untransformed cells is a much greater spread of chromosome numbers in the transformed cells and a general shift of the mean and modal chromosome numbers to lower values than that of untransformed cells. The mean chromosome number represents the average number of chromosomes per metaphase whereas the mode represents the chromosome number most often counted in the metaphases examined. Only a small number of cell lines show modal chromosome numbers greater than the untransformed cells and three of these four cell lines are tumour cell lines. Several more cell lines show mean chromosome numbers greater than the untransformed cells and this can be explained by the influence of a small fraction of these cells (showing over a hundred chromosomes per metaphase) on the overall mean. The modal chromosome number of the untransformed cells lies between that of the spontaneous focus and its tumour cells while the mean chromosome number of both the spontaneous focus and tumour cell lines is greater than that of the untransformed C3H10T½ cells. Since the actual number of chromosomes per cell is likely to be more relevant to the biological effects of the radiation than the mean number of chromosomes per cell in a cell population this discussion concentrates on the differences found between the modal chromosome numbers and the distribution of the chromosome numbers rather than the mean chromosome values.

Data on the radiation - induced foci and tumour cells are examined firstly by comparison of the foci (combined data of tumourigenic and non - tumourigenic foci) then comparison of the tumourigenic foci, followed by examination of the tumour cells. These data are presented in each comparison as the combined data of X-rays and alpha-particles and then compared according to radiation type.

Comparison of focus data

The first comparison is between the different focus categories using the combined data of the tumourigenic and non - tumourigenic foci. The modal chromosome number of the radiation induced (+) and (X) foci (combined data of X-

rays and alpha-particles) is slightly greater than that of the (X/+) and (-) foci. Examination of these data according to radiation type shows the alpha-particle induced (X) foci have a greater modal chromosome number than the other alpha-particle induced foci while the modes of the X-ray induced (+), (X/+) and (X) foci are similar to each other and higher than that of the X-ray induced (-) foci. The distribution of chromosome number for the (-) foci is different to that of the other X-ray induced foci showing two groups of metaphases, one with chromosome numbers in the range of 60 to 74 chromosomes per metaphase and the other showing higher chromosome numbers in the range of 100 to 114 chromosomes per metaphase.

Comparison of the data for the tumourigenic foci shows the radiation induced (+), (X/+), and (X) foci (combined data of X-rays and alpha-particles) have similar modal chromosome numbers while the (-) foci have a lower mode. Two X-ray induced tumourigenic (-) focus cell lines were examined and the lower modal chromosome number is mostly contributed by one of the cell lines while the higher modal chromosome number is mostly contributed by the other cell line. This highlights the heterogeneity of the (-) foci compared to any of the other focus categories. A similar pattern was observed for the X-ray induced tumourigenic foci as outlined in the previous paragraph where the modal chromosome number of the X-ray induced (+), (X/+) and (X) foci are similar to each other and greater than that of the X-ray induced (-) foci. Comparison of the alpha-particle induced foci shows the modes of the (+) and (-) foci are similar and higher than that of the (X/+) foci (no tumourigenic (X) foci were examined).

Comparison of tumour cells

Examination of the tumour cells from the radiation - induced foci (combined data of X-rays and alpha-particles) shows a much broader distribution of chromosome numbers with more metaphases containing higher numbers of chromosomes than is observed for the foci. Tumour cells from the (-) foci especially show a wide distribution of chromosome numbers. The modal chromosome number of the tumour cells from the radiation - induced (+) cells is greater than that of the other categories which show the same range of modal chromosome numbers. Examination of the

tumour cells from the X-ray induced foci displays that the (X) and (X/+) cells present two groups of metaphases, one with chromosome numbers in the range of 60 to 69 chromosomes per metaphase and the other in the range of 115 to 124 chromosomes per metaphase. The modal chromosome number of the (X/+) cells (120 to 124 chromosomes per metaphase) is considerably greater than that of the other categories with the tumour cells from the (-) foci having the lowest mode (60 to 64 chromosomes per metaphase). Examination of the tumour cells from the alpha-particle induced foci shows that the modal number of the (+) cells is greater than that of the (X/+) and (-) cells which are both in the same range. No data are available for tumour cells from alpha-particle induced (X) foci. The (-) cells seem to consist of two groups, one set with chromosome numbers in the range of 65 to 74 chromosomes per metaphase and the other set with higher chromosome numbers in the range of 110 to 124 chromosomes per metaphase. As previously described for the X-ray induced (-) foci, the two groups of tumour cells from the alpha-particle induced (-) foci derive mainly from the combination of cell lines with different modal chromosome numbers, in this case two cell lines contributed most to the lower modal chromosome number and the other cell line in the group contributed mainly to the higher modal chromosome number.

Comparison of foci and tumour cells

Examination of the different focus categories comparing foci and tumour cells induced by X-rays and alpha-particles shows that the (+) cells have the narrowest range of chromosome numbers with few metaphases having more than eighty - four chromosomes. No major differences are noted in the modal chromosome number of the (+) foci versus the tumour cells or the X-ray versus alpha-particle comparisons. The (X/+) tumour cells show a wider distribution of chromosome numbers than is seen for the (+) cells and the tumour cells from the X-ray induced (X/+) focus have a considerably higher mode (120 to 124 chromosomes per metaphase) than its corresponding focus (70 to 74 chromosomes per metaphase). No differences are found in the mode of the alpha-particle induced (X/+) focus and its corresponding tumour cells. The modal chromosome values of the alpha-particle induced foci and tumour

cells are lower than those of the corresponding X-ray induced foci and tumour cells. Data on an X-ray induced focus and corresponding tumour cells (no alpha-particle induced (X) focus or tumour cells) in the (X) category show the mode of the focus is slightly higher than that of the tumour cells with few focus cell metaphases containing greater than 84 chromosomes. A notable increase of metaphases with chromosome numbers in the 115 to 129 range is observed for the tumour cells compared to the focus. The distribution of the chromosome numbers for the (-) foci and tumour cells shows the widest range of all the focus categories with both the X-ray induced foci and the tumour cells from the alpha-particle induced foci showing two groups of cells, one with the mode in the range of 60 to 74 chromosomes per metaphase and the other with the mode in the range of 100 to 120 chromosomes per metaphase, which as explained above is due to the heterogeneous nature of different cell lines categorised as (-) foci. The modal chromosome number is highest for the alpha-particle induced foci (70 to 74 chromosomes per metaphase) and lowest for the tumour cells from the X-ray induced (-) foci (60 to 64 chromosomes per metaphase) with the modes of the X-ray induced foci and the tumour cells from the alpha-particle induced (-) foci between these ranges.

Loss or gain of DNA in transformation

The original publication on the C3H10T½ cell line reported that untransformed cells are hypertetraploid with a small proportion of cells in the octaploid range (Reznikoff *et al.* 1973). Furthermore it was stated that the modal chromosome number for transformed cells remains near tetraploid with a greater distribution of chromosome number around the mode. The number of octaploid cells was increased in the transformed cells examined by Reznikoff *et al.* (1973). The modal chromosome number in this thesis for untransformed C3H10T½ cells is lower than reported by Reznikoff *et al.* (1973) (77 versus 81 chromosomes per metaphase) with a move of modal chromosome numbers to lower values in transformed cells. Smith *et al.* (1993) analysed the DNA content of chemically transformed cells and found that eighty percent of the clones examined lost considerable amounts of DNA even in the passages in culture required to establish the cell lines. The reduced DNA content of

transformed C3H10T½ cells relative to the untransformed cells was observed in both tumourigenic and non - tumourigenic cell clones although the tumourigenic clones lost greater quantities of DNA. The same study also reported that some of the tumourigenic clones gained genomic material (approximately 1.5 times that of the untransformed C3H10T½ cells).

Smith *et al.* (1993) studied the combination of particular phenotypes of transformed C3H10T½ cells, including morphological changes, ability to grow in agar and reconstruct foci, higher saturation densities and longer doubling times as well as DNA changes to assess which combination most accurately predicted the tumourigenicity of the transformed focus cell lines examined. They postulated that, although morphological changes (which result in focus formation) occur with the frequency of mutation of a single gene, the acquisition of the other transformation phenotypes listed above appear to occur in the morphologically transformed cells with frequencies higher than would be typical of a complex phenotype that needs multiple single gene mutations. The high frequency and variability of expression of the transformation phenotypes in the morphologically transformed cells suggested to the authors the possibility of an epigenetic mechanism which results in quantitative changes in the expression of structurally unaltered genes rather than alterations in gene products due to gene mutations. One possibility is that an early step in transformation and / or carcinogenesis is the activation of a gene which allows mutations to occur at much higher frequencies than normal, however Smith *et al.* (1993) also proposed that instead of increased gene mutation rates causing the acquisition of complex phenotypes their results indicated a possible alternative mechanism involving a rapid loss of DNA by the morphologically transformed cells. Their proposal is that the initial event leading to morphological transformation could be a critical genetic alteration which results in a rapidly occurring genomic instability which is correlated with epigenetic changes in gene expression resulting in the acquisition of additional transformation phenotypes and tumourigenicity. The Smith study used chemical carcinogens to transform the C3H10T½ cells although the same proposal could be applied to radiation transformed C3H10T½ cells. Genomic rearrangements with loss of a specific DNA region have been observed in X-ray transformed C3H10T½ cells

(Paquette *et al.* 1992, 1994). The loss of DNA is also observed in the data presented in this section where most of the foci and tumour cells have lower modes than the untransformed cells.

Polyploidy state of transformed C3H10T½ cells

No major trends are apparent in the comparison of the different focus / tumour cell categories (or between the X-rays and alpha-particles) with the possible exception that generally the (-) foci and tumour cells have lower modes than the other categories. In conjunction with this is the presence of two groups of cells in the (-) cell lines (especially the X-ray induced foci and the tumour cells from the alpha-particle induced foci) with one group of cells having chromosome numbers in a slightly lower range to that of the untransformed cells and the other group of cells having chromosome numbers greater than 100 chromosomes per metaphase. Two possibilities which may explain these data compared to the other focus / tumour cell categories are firstly that the phenomenon is unique to the (-) cells or secondly that the other foci and tumour cells showed the same at some stage but have now reverted to a lower more stable modal chromosome number. The second possibility seems more likely and is supported by studies of X-ray transformed C3H10T½ cell lines carried out by Crompton *et al.* (1994). Their examination of the transformed foci revealed that most of the clones showed multiple ploidy states, even in clones originally isolated from a single cell. A reduction in the number of cells showing the higher ploidy states often occurred with cell growth and passage, resulting in the reversion of the population to the hypertetraploid state of the parent cells (Crompton *et al.* 1994). The conclusion that Crompton *et al.* came to was that exposure to radiation can induce, in association with morphological transformation a heritable, genomically labile state and in association with the genome instability the C3H10T½ cells have a tendency to undergo polyploidisation. The ploidy of the cells could result from an aberrant anaphase in the division of the cells (Crompton *et al.* 1994). Furthermore since Smith *et al.* (1993) reported no significant polyploidy in the chemically transformed C3H10T½ foci they examined, Crompton *et al.* surmised that the polyploidy appears to be an action of the radiation. However an earlier study reported that both

untransformed and chemically transformed C3H10T½ cells contain small subpopulations of higher ploidy cells and that although the higher ploidy population are unlikely to be responsible for the transformation, the appearance of polyploid subpopulations may however accompany chromosome instability in the transformed cells (Saxholm and Digernes 1980).

Although the polyploidy reported by Crompton *et al.* (1994) is not repeated in the studies in this thesis, a considerable spread of chromosome numbers is observed in the metaphases of the transformed C3H10T½ foci induced by both X-rays and alpha-particles compared to the untransformed cells. Furthermore this distribution is more pronounced in the tumour cells. It must be remembered that the focus cell population is an impure mix of cells while the tumour cells are a purer collection of transformed, tumourigenic cells and the increased number of metaphases with higher chromosome numbers in the tumour cells may be indicative of genome instability induced by the radiation. The phenomenon does not appear to be unique to the high- or low- LET radiation. The apparent lack of a second population of cells with greater than 100 chromosomes per metaphase in some categories of foci and tumour cells examined in this thesis may be, as reported by Crompton *et al.* (1994), due to a reversion of the population to the parental state with continued growth of the cells in culture. Data on the X-ray induced (-) foci and tumour cells could be seen to support this as the foci show a second population of cells with chromosome numbers in the range of 100 to 114 chromosomes per metaphase, whereas the tumour cells show a similar population which however have a lower number of chromosomes (80 to 99 chromosomes per metaphase) which nevertheless are still higher than the actual mode of 60 to 64 chromosomes per metaphase. This is the case for both focus cell lines examined in this category compared to their tumour cell lines. One may speculate that with further culture the chromosome numbers of the tumour cells would all end up in the range of 60 to 80 chromosomes per metaphase, as the metaphases with higher chromosome numbers are lost from the population. Interestingly the (+) focus and tumour cells show the narrowest distribution of chromosome numbers for both X-rays and alpha-particles and one might speculate following on the above train of thought that these cells have now attained relatively stable genomes and have reverted closer

to the range of chromosome numbers of the untransformed parent C3H10T½ cells. However the above speculation is not supported by the X-ray induced (X/+) and (X) data or the alpha-particle induced (-) data. Tumour cells from the X-ray induced (X/+) foci and the alpha-particle induced (-) foci show a second population of metaphases with high chromosome numbers while the foci do not and the (X) focus and tumour cells both show subpopulations of cells with 115 to 119 chromosomes per metaphase indicating a stable subpopulation. These data support the idea of long - lived damage after radiation exposure as this phenomenon of genome instability is still evident after focus isolation, development of the focus cell line, tumourigenicity testing, development of the tumour cell line and subculture of the cells.

Robertsonian translocations

During the course of the cytogenetic studies the presence of Robertsonian chromosomes was observed in some metaphases. These Robertsonian chromosomes are not unique to the radiation treated cells as a background level of six percent is observed in the untransformed cells and few cell lines have levels greater than this. The only exceptions are the tumourigenic and non - tumourigenic X-ray induced (X/+) foci and the tumour cells from the X-ray induced (+) foci which all display percentage metaphases with Robertsonian chromosomes greater than the level observed in the untransformed C3H10T½ cells. Comparison of the percentage metaphases with Robertsonian chromosomes within each focus category shows that for the (+) foci the percentage decreases in the order non - tumourigenic alpha-particle induced foci to tumourigenic X-ray induced foci, then the tumourigenic alpha-particle induced foci and spontaneous focus. In the corresponding tumour cells the percentage metaphases with Robertsonian chromosomes is highest for the X-rays and lowest for the alpha-particles with the level of the spontaneous cells in between the two radiation types. The level of Robertsonian chromosomes in the (X/+) foci is greater for the X-ray induced foci (tumourigenic and non - tumourigenic) than for the alpha-particle equivalent, with no differences observed between the tumour cells. Few Robertsonian chromosomes appeared in the metaphases of the (X) foci or tumour cells. A greater percentage of metaphases of the alpha-particle induced (-) foci contain Robertsonian chromosome

compared to the X-ray induced equivalent with no differences observed between the tumour cells. Comparison of the foci and their tumour cells shows the spontaneous and X-ray induced (+) foci have lower levels of Robertsonian chromosomes than the corresponding tumour cells while the (X/+) foci (X-ray and alpha-particle induced) have higher levels than their tumour cells. Foci and tumour cells of the alpha-particle induced (+) and (-) foci and the X-ray induced (X) and (-) foci show similar percentages of metaphases with Robertsonian chromosomes. Thus one can surmise that Robertsonian chromosomes are not radiation- specific nor are they unique to transformed or tumourigenic cells and X-rays generally produced more Robertsonian chromosomes than alpha-particles.

Robertsonian chromosomes are the most common structural chromosome abnormality in humans, one in a thousand in the general population (Wolff *et al.* 1992), can occur spontaneously and are not thought to be specifically induced by ionising radiation, this is supported by the high percentage of Robertsonian chromosomes found in the untransformed C3H10T½ cells examined in this thesis and in other studies (Bryant and Riches 1989, Durante *et al.* 1992, 1994). Robertsonian chromosomes result from fusion of acrocentric chromosomes either by centromeric fusion or whole arm reciprocal translocations, which by definition would involve the loss of one centromere. The translocations may be monocentric or dicentric depending on the site of recombination. In dicentrics the second centromere is often inactivated and the translocated chromosome can be stably inherited (Niebuhr *et al.* 1972, Therman *et al.* 1989, Gravholt *et al.* 1992). Since it is generally assumed that no direct adhesion of chromosome ends is possible centric fusion involves breakage in the very short arms of the two acrocentric chromosomes followed by the fusion of the two long parts into a single chromosome with the loss of the two small fragments. There have been reports of no significant loss of DNA during Robertsonian fusion in several cell lines examined (Redi *et al.* 1986, Schubert *et al.* 1992). The totally acrocentric constitution of the mouse karyotype and the relative uniformity of the satellite DNA would allow random acrocentric fusions (Redi *et al.* 1986, 1990). Others propose that Robertsonian rearrangements are not translocations due to the customary chromosome breakage process but rather due to recombination events between areas of homology

(Hecht *et al.* 1988, Durante *et al.* 1992). Thus the higher number of Robertsonian translocations induced by X-rays compared to alpha-particles may be explained by studies which show that X-rays induce more exchanges than breaks in chromosomes whereas a higher percentage of chromosome breaks is observed after alpha-particles (Durante *et al.* 1992). The same study also reported that both types of radiation produced a significant increase in the number of Robertsonian translocations over the background level (observed at the first mitosis after irradiation), and the increase was related to cell cycle phase and radiation dose (Durante *et al.* 1994). Since these translocations are expected to be dicentrics many may be unstable and thus would be lost in subsequent cell divisions unless one of the centromeres become inactive and thus the translocation becomes more stable as has been reported for Robertsonian translocations (Niebuhr *et al.* 1972, Therman *et al.* 1989, Gravholt *et al.* 1992). The Robertsonian chromosomes observed in the focus and tumour cells may be stably transmitted through cell divisions or an equilibrium may exist with some Robertsonian chromosomes being lost and new ones being generated during cell division

Proto-oncogenes and tumour suppressor genes

The next part of this discussion concentrates on the possible implications of the loss of chromosomes observed in the radiation treated cells and also the possible implications of the gain of genomic material noted in some focus and tumour cells. Initiation and / or development of carcinogenesis may occur through the activation of proto-oncogenes or the inactivation of tumour suppressor genes (UNSCEAR 1993). The activation of proto-oncogenes may occur through point mutation, chromosomal translocation and juxtaposition with another DNA sequence or though gene amplification while tumour suppressor genes may be inactivated by deletions or mutations which lead to the loss of function of the suppressor genes (UNSCEAR 1993).

Numerous studies have been done to try and identify oncogenes which may be involved in the transformation of C3H10T½ cells. X-ray transformed cell lines examined for oncogene mutations showed no gross rearrangements or amplifications of v-Ha-*ras*, v-Ki-*ras*, N-*ras*, v-*myc*, v-*raf*, v-*src*, v-*fes*, v-*abl*, v-*mos*, v-*erb*-A, v-*erb*-B,

v-*myb*, *neu*, *trk*, *fms*, v-*fos*, *mdm2* or v-*sis* oncogenes (Shuin *et al.* 1986, Borek *et al.* 1987, Krolewski *et al.* 1989, 1994, 1995, Privitera *et al.* 1990, Thomas and Guernsey 1991). An enhanced level of c-*myc* protein (without structural change in the gene), decreased level of c-*fos*, structural changes in the p53 gene, enhanced mRNA levels of *raf* and mutated c-K-*ras* genes have been reported in some transformed C3H10T½ cell lines (Shuin *et al.* 1986, Chen and Herschmann 1988, Thomas and Guernsey 1991, Leuthauser *et al.* 1992, Smith *et al.* 1993, Coleman *et al.* 1994, Krolewski and Little 1993, 1994). It is not known whether the mutation in the *ras* gene is involved in transformation since the mutated allele appears not to be expressed and was not enhanced in all the transformed C3H10T½ cell lines examined (Thomas and Guernsey 1991). Transfection of C3H10T½ cells with an exogenous c-*myc* oncogene greatly enhances the sensitivity to transformation (Sorrentino *et al.* 1987), thus *c-myc* appears to act as an accomplice in transformation, though not necessarily involved in the initiation of transformation. An overexpression of the mutant p53 protein in a number of chemically transformed C3H10T½ cell lines correlated well with the tumourigenicity of these cells as did the coordinated overexpression of c-K-*ras*, c-N-*ras*, and transforming growth factor B (Coleman *et al.* 1994).

The results to date indicate that the coordinated overexpression of a number of oncogenes working together assists in the progression of chemically transformed C3H10T½ cells through transformation to tumourigenicity and some studies would seem to indicate that radiation transformation is not due to one single oncogene activation but rather a combined effort of a number of oncogenes (Shuin *et al.* 1986, Borek *et al.* 1987). Since the general impression of the above studies seems to be that overexpression of the various oncogenes may be involved in transformation / tumourigenesis rather than mutations within these oncogenes, this would seem to indicate that radiation treatment acts by affecting the regulation of the transcription of these oncogenes. One can speculate that both populations of transformed C3H10T½ cells outlined in the cytogenetic data presented in the results section could increase the transcription of the proto-oncogenes. Cells which have lost DNA compared to the untransformed C3H10T½ cells may also have lost important copies of control genes for the regulation of transcription of proto-oncogenes while cells which have gained

genomic material may have also gained copies of positive control genes for transcription thus increasing the signal to the transcription genes and consequently increasing transcription of oncogenes.

Genomic instability

The final comment in this section is on the ability of radiation to induce genomic instability. Several reports advocate this capability of radiation in several cell systems with the instability mostly characterised by the appearance of non - clonal aberrations within a clonal population several cell generations after the initial radiation exposure (for example, Kronenberg 1994, Kadhim *et al.* 1992, 1994, 1995, Sabatier *et al.* 1992, Marder and Morgan 1993, review of data in UNSCEAR 1993). High- LET radiation seems to be a more effective inducer of instability than X-rays (Kadhim *et al.* 1992, 1994, 1995) although instability has been shown to be induced by X-rays in some cell systems (Holmberg *et al.* 1995, Kano and Little 1984). Griffin *et al.* (1995) reported on the range of complex aberrations (defined as at least three breaks in two chromosomes) produced by X-rays and alpha-particles in primary human fibroblasts and stated that they would expect the complexes to be transmissible to future cell generations and that a higher proportion of the complexes produced by alpha-particle irradiation are potentially transmissible and these lesions could lead to transformation and / or chromosomal instability. The same study also deemed that since complexes are more likely to develop after high- LET radiation exposure the risk of delayed genetic effects is greater after exposure to high- rather than low- LET radiation (Griffin *et al.* 1995). The genomic instability could occur by a number of mechanisms such as a genome - wide process where the mutant enzymes in DNA replication or repair could cause alterations in the DNA or the process may involve a specific class of genes which monitor genome integrity and ensure cell cycles and DNA repair proceed correctly. While many authors favour the genome - wide process especially with the wide distribution of damage radiation can cause, some authors favour specific damage to the DNA causing the genomic instability (Sabatier *et al.* 1992, Paquette and Little 1992, 1994). In one of these studies the analysis of genomic rearrangements in X-ray transformed C3H10T½ cells using four multilocus and multiallele probes (to

detect different minisatellite families in the DNA) revealed rearrangements could only be detected with one probe suggesting a specific target for the X-rays (Paquette and Little 1992, 1994).

The data presented in this section indicate genome instability is induced by both high- and low- LET radiation in C3H10T½ cells and the implications of this instability for the other transformation parameters discussed in previous sections of this chapter will be examined in the next section.

Summary

There is a notably greater spread of chromosome numbers in the transformed cells and a general shift of the mean and modal chromosome numbers to lower values than that of untransformed cells. Only a small number of cell lines show modal chromosome numbers greater than the untransformed cells and three of these four cell lines are tumour cell lines. No major trends are apparent in the comparison of the different focus / tumour cell categories (or between X-rays and alpha-particles) with the possible exception that generally the (-) foci and tumour cells have lower modes than the other categories. The highest modal chromosome number exists for the tumour cells from the X-ray induced (X/+) foci (120 to 124 chromosomes per metaphase) which is approximately double that of other foci and tumour cell categories. Tumour cells show a much broader distribution of chromosome numbers with more metaphases containing higher numbers of chromosomes than is observed for the foci. Of all the focus and tumour cell categories the widest distribution of the chromosome numbers is seen for the (-) foci and tumour cells. A number of cell lines show two populations of metaphases one with chromosome numbers in the range of 60 to 74 chromosomes per metaphase and the other population showing greater than 100 chromosomes per metaphase. These cell lines are those from the X-ray induced (-) foci and the tumour cells from the X-ray induced (X) and (X/+) foci and the alpha-particle induced (-) foci.

The presence of Robertsonian chromosomes is not unique to the radiation treated cells as a background level is observed in the untransformed cells and few cell lines have levels greater than this. The only exceptions are the tumourigenic and non - tumourigenic X-ray induced (X/+) foci and the tumour cells from the X-ray induced (+) foci.

Percentage metaphases with chromosome numbers in the following ranges							
	Radiation -induced foci				Untranformed cells	Spontaneous focus	Tumour cells from spontaneous focus
Chromosome numbers	(+) n=13	(X/+) n=6	(X) n=3	(-) n=7		(+) n=1	(+) n=1
< 45	0.2	0.5	0	0	0	0	1
45 - 49	0.2	0.3	0	0.3	0	0	0
50 - 54	0.9	1.5	0	1.4	0	1.4	1
55 - 59	3.8	6.2	0.3	5.9	0.9	5.6	0
60 - 64	12	17.3	4.3	15.6	1.8	11.2	0
65 - 69	24.4	29.2	18	25	13.5	18.2	0
70 - 74	32.4	26.2	35.7	24.1	32.1	13.9	0
75 - 79	15.7	12.2	31	9.7	45.6	7	0
80 - 84	3	3	7.4	2	6.3	1.4	1
85 - 89	0.4	0.7	0.3	0.6	0	0	3
90 - 94	0.6	0	0.7	0.9	0	1.4	9
95 - 99	0.2	0	0.7	1.4	0	0	21
100 - 104	0.3	0.7	0.3	3	0	4.2	23
105 - 109	0.5	0.2	0.3	4.1	0	4.2	6
110 - 114	0.4	0.5	0	2.4	0	4.2	6
115 - 119	0.5	0.2	1	1.3	0	1.4	0
120 - 124	1	0.2	0.3	0.6	0	8.4	10
125 - 129	1.2	0.2	0	0.4	0	7	4
130 - 134	0.7	0.7	0	0.3	0	7	6
135 - 139	0.8	0.2	0	0.4	0	2.8	6
> 140	0.9	0.3	0	0.6	0	1.4	3
Robertsonian chromosomes	4.6	13.3	0.3	3.7	6.3	1.4	4

Appendix 5.5.1. Table shows the range of chromosome numbers of the radiation induced foci (combined data of tumourigenic and non - tumourigenic X-ray and alpha-particle induced foci), untransformed cells and the spontaneous focus and tumour cells. These data are also presented in figures 5.5.2, 5.5.3 (a) and 5.5.7.

Percentage metaphases with chromosome numbers in the following ranges								
	X-ray induced foci				Alpha-particle induced foci			
Chromosome numbers	(+) n=8	(X/+) n=2	(X) n=2	(-) n=3	(+) n=5	(X/+) n=4	(X) n=1	(-) n=4
< 45	0.3	0	0	0	0.2	0.8	0	0
45 - 49	0.3	0.5	0	0.3	0	0.3	0	0.3
50 - 54	1.4	0	0	1.7	0	2.3	0	1.3
55 - 59	5	3	0.5	7.5	2	7.8	0	4.8
60 - 64	13.9	7	5.4	18.3	8.9	22.5	2	13.5
65 - 69	24.3	21.5	23	23.4	24.7	33	8	26.3
70 - 74	30.3	38	39.1	10.7	35.7	20.3	29	34.3
75 - 79	15.6	21.5	19.5	6.3	15.9	7.5	54	12.3
80 - 84	3.4	6.5	8	2.3	2.3	1.3	6	1.8
85 - 89	0.4	1	0.5	0.7	0.4	0.5	0	0.5
90 - 94	0.9	0	1	2	0.2	0	0	0
95 - 99	0.1	0	0.5	3.3	0.4	0	1	0
100 - 104	0.1	0	0.5	6.7	0.6	1	0	0.3
105 - 109	0.5	0	0.5	9	0.4	0.3	0	0.5
110 - 114	0.3	0	0	4.3	0.5	0.8	0	1
115 - 119	0.4	0	1.5	1.3	0.8	0.3	0	1.3
120 - 124	0.5	0	0.5	1.3	1.7	0.3	0	0
125 - 129	1	0	0	0.3	1.6	0.3	0	0.5
130 - 134	0.1	0	0	0	1.6	1	0	0.5
135 - 139	0.9	0	0	0.3	0.7	0.3	0	0.5
> 140	0.6	1	0	0.3	1.5	0	0	0.8
Robertsonian chromosomes	4.9	34.5	0.5	0.7	4.3	2.8	0	6

Appendix 5.5.2. Table shows the range of chromosome numbers of the X-ray and alpha-particle induced foci (combined data of tumourigenic and non - tumourigenic foci). These data are also presented in figures 5.5.3 (b) and (c).

Percentage metaphases with chromosome numbers in the following ranges								
	Radiation induced foci				Tumour cells from radiation induced foci			
Chromosome numbers	(+) n=11	(X/+) n=3	(X) n=1	(-) n=5	(+) n=11	(X/+) n=3	(X) n=1	(-) n=5
< 45	0.2	0	0	0	0	0	0	0
45 - 49	0.2	0	0	0.4	0.3	0.3	0	0.5
50 - 54	1	1.7	0	0.6	2.2	2.7	5	3
55 - 59	4.3	3.7	1	5.7	5.3	7.3	11	6.1
60 - 64	12.4	16	8.8	17.2	12.7	16.7	22	12.2
65 - 69	24.6	23	35.9	24.8	26.3	28.7	29	16.9
70 - 74	32.8	31.3	38.8	23.6	34.8	14.3	11	13.6
75 - 79	16.5	13	6.8	8.2	12.2	4.7	1	12.1
80 - 84	3.3	4.7	3	1.6	2.3	1.3	1	2.9
85 - 89	0.5	1.3	0	0.4	0.3	1.7	0	4.3
90 - 94	0.6	0	0	1.2	0.5	0.7	1	2.4
95 - 99	0.2	0	1	2	0.4	2.3	0	3.3
100 - 104	0.2	1	0	4	0.3	1	2	2.3
105 - 109	0.4	0.3	1	5.4	0.2	3	0	4.6
110 - 114	0.2	1	0	2.6	0.3	2.3	0	4.3
115 - 119	0.3	0.3	2.9	1.4	0.2	2.3	7	5.6
120 - 124	0.5	0.3	1	0.8	0.4	5	5	2.4
125 - 129	0.8	0.3	0	0.2	0.2	1.3	3	1.3
130 - 134	0.1	1.3	0	0	0.3	1.7	0	0.7
135 - 139	0.6	0	0	0	0.2	1.7	1	0.5
> 140	0.5	0.7	0	0	0.7	1	1	0.9
Robertsonian chromosomes	4	19.7	1	3.4	11.9	1.7	0	3.4

Appendix 5.5.3. Table shows the range of chromosome numbers of the radiation induced foci (combined data of tumourigenic X-ray and alpha-particle induced foci) and tumour cells. These data are also presented in figures 5.5.4 (a) and 5.5.5 (a).

Percentage metaphases with chromosome numbers in the following ranges								
	X-ray induced foci				Tumour cells from X-ray induced foci			
Chromosome numbers	(+) n=8	(X/+) n=1	(X) n=1	(-) n=2	(+) n=8	(X/+) n=1	(X) n=1	(-) n=2
< 45	0.3	0	0	0	0	0	0	0
45 - 49	0.3	0	0	0.5	0.4	0	0	1.3
50 - 54	1.4	0	0	0.5	2.7	1	5	7.4
55 - 59	5	1	1	8.2	4.7	6	11	14.8
60 - 64	13.9	1	8.8	20.5	13.1	8	22	24
65 - 69	24.3	13	35.9	20.6	28.3	9	29	7.2
70 - 74	30.3	45	38.8	4	35.6	7	11	2.3
75 - 79	15.6	26	6.8	1.9	9.6	3	1	10.4
80 - 84	3.4	10	3	1	0.6	1	1	3.9
85 - 89	0.4	2	0	0.5	0.3	3	0	9.1
90 - 94	0.9	0	0	3	0.5	2	1	5.2
95 - 99	0.1	0	1	5	0.4	7	0	6.2
100 - 104	0.1	0	0	10	0.5	3	2	2.6
105 - 109	0.5	0	1	13.5	0.3	8	0	2.3
110 - 114	0.3	0	0	6.4	0.4	7	0	2
115 - 119	0.4	0	2.9	2	0.3	6	7	1
120 - 124	0.5	0	1	2	0.6	14	5	0.5
125 - 129	1	0	0	0.5	0.3	4	3	0
130 - 134	0.1	0	0	0	0.4	3	0	0
135 - 139	0.9	0	0	0	0.3	5	1	0
> 140	0.6	2	0	0	1	3	1	0
Robertsonian chromosomes	4.9	51	1	1	16.1	2	0	2.8

Appendix 5.5.4. Table shows the range of chromosome numbers of the tumourigenic X-ray induced foci and tumour cells. These data are also presented in figures 5.5.4 (b), 5.5.5 (b), 5.5.6 (a,b,c,d) and 5.5.7.

Percentage metaphases with chromosome numbers in the following ranges								
	Alpha-particle induced foci				Tumour cells from alpha - particle induced foci			
Chromosome numbers	(+) n=3	(X/+) n=2	(X)	(-) n=3	(+) n=3	(X/+) n=2	(X)	(-) n=3
< 45	0	0		0	0	0		0
45 - 49	0	0		0.3	0	0.5		0
50 - 54	0	2.5		0.7	0.7	3.5		0
55 - 59	2.3	5		4	6.7	8		0.3
60 - 64	8.5	23.5		15	11.7	21		4.3
65 - 69	25.6	28		27.7	21	38.5		23.3
70 - 74	39.4	24.5		36.7	32.7	18		21.2
75 - 79	18.9	6.5		12.3	19	5.5		13.3
80 - 84	2.9	2		2	6.7	1.5		2.3
85 - 89	0.7	1		0.3	0.3	1		1.1
90 - 94	0	0		0	0.7	0		0.4
95 - 99	0.3	0		0	0.3	0		1.3
100 - 104	0.3	1.5		0	0	0		2.1
105 - 109	0	0.5		0	0	0.5		6.2
110 - 114	0	1.5		0	0	0		5.8
115 - 119	0	0.5		1	0	0.5		8.8
120 - 124	0.7	0.5		0	0	0.5		3.8
125 - 129	0.3	0.5		0	0	0		2.1
130 - 134	0	2		0	0.3	1		1.1
135 - 139	0	0		0	0	0		0.9
> 140	0	0		0	0	0		1.5
Robertsonian chromosomes	1.6	4		5	0.7	1.5		3.8

Appendix 5.5.5. Table shows the range of chromosome numbers of the tumourigenic alpha-particle induced foci and tumour cells. These data are also presented in figures 5.5.4 (c), 5.5.5 (c), 5.5.6 (a,b,c,d) and 5.5.7.

Section 5.6.

Discussion of transformation properties

The C3H10T½ transformation assay is a well established assay used to examine carcinogenic properties of a vast array of agents including different types of radiation and chemicals. The assay involves the production of foci which are classified as positively or negatively transformed and the positively transformed foci are then used to calculate transformation frequencies. These transformation frequencies have been and continue to be widely used for comparison of different doses and types of radiation as well as chemicals. It is apparent that a very important aspect of this assay is to define and clarify what are the important criteria for identification of positively transformed foci.

In this thesis a number of radiation-induced C3H10T½ foci were isolated, expanded as cell lines and a number of the cell properties determined. The objectives of the work were twofold, firstly to examine qualitative differences in properties of the foci when produced by high- or low- LET radiation and secondly to compare a sample of the many varieties of foci that are seen to determine if different focus phenotypes have properties that differ from other phenotypes or whether such properties of transformed cells are independent of focus categorisation.

The four categories of foci examined were (+), (X/+), (X) and (-), these cell lines were examined for tumourigenicity, ability to reconstruct foci on two different types of monolayers, cytogenetic changes as well as changes in growth parameters such as lag times, doubling times and saturation densities. These have all been discussed in detail above and a synopsis of the main findings and pertinent conclusions are presented here.

Summary of cytogenetics data

The cytogenetic studies presented a greater spread of chromosome numbers in transformed cells and a general shift of the mean and modal chromosome numbers to lower values than that of untransformed cells. This applied for the four focus categories induced by both X-rays and alpha-particles though generally the (-)

transformed foci and tumour cells had lower modal chromosomal numbers than the other categories. Tumour cells showed a broader distribution of chromosome numbers containing more metaphases with higher numbers of chromosomes per metaphase than the foci or untransformed cells. The most extreme example was the tumour cells from the X-ray induced (X/+) foci which had a modal chromosome number of one hundred and twenty chromosomes per metaphase which is approximately twice that of the other focus categories. A notable feature in some cell lines was the appearance of two groups of metaphases one with chromosome numbers in the range observed for most of the cell lines of sixty to seventy four chromosomes per metaphase and the other group showing greater than one hundred chromosomes per metaphase. These cell lines were developed from the (-) foci (X-ray and alpha-particle induced foci) and tumour cells derived from the X-ray induced (X) and (X/+) foci. The presence of metacentric chromosomes (Robertsonian translocations) was not unique to the radiation treated cells as most of the cell lines showed fewer of these chromosomes than observed in the untransformed cells. Nevertheless the X-ray induced foci and tumour cells generally produced more Robertsonian chromosomes than the alpha-particle induced equivalent with the exception of the (-) foci and tumour cells where the reverse occurred.

Summary of growth parameters

No differences were found in the lag or doubling times of the cells irrespective of focus category, radiation type, or transformed or tumourigenic nature of the cells. Differences were noted between the cell lines in the saturation densities which were generally higher when cells were grown in standard serum conditions compared to reduced growth conditions. Most of the foci produced saturation densities comparable or slightly lower than those of the untransformed cells while the tumour cells had consistently lower cell densities. Generally for both foci and tumour cells, where differences occurred between X-ray and alpha-particle treated cells the differences were higher saturation densities for the X-ray treated cells rather than the alpha-particle treated cells although the reverse was true for the (-) cells.

Summary of focus reconstruction studies

In the focus reconstruction studies cells from the alpha-particle induced foci generally produced higher frequencies of reconstructed foci per viable cell (but lower total numbers of foci) than the X-ray equivalent while the reverse was true for the tumour cells. Foci (X-ray and alpha-particle induced) generally produced lower focus frequencies than the corresponding tumour cells. Of the different focus categories cells from (+) foci and derived tumour cells were capable of reproducing all other categories of reconstructed foci and did so on almost all occasions while cells from (X) foci and derived tumour cells tended to reconstruct (X) or (-) foci. Cells isolated from (X/+) foci and derived tumour cells generally reconstructed (+), (X) or (-) foci in preference to reconstructed (X/+) foci while the cells from (-) foci reconstructed (+) and (-) foci only.

Summary of tumourigenicity data

In all categories of foci a higher proportion of X-ray induced foci were tumourigenic than non-tumourigenic while most of the alpha-particle induced foci were non - tumourigenic. X-ray induced foci generally produced tumours earlier and needed less time for all tumours to become apparent than the alpha-particle induced foci. Cells from (X/+) foci produced the fastest growing tumours while the cells from (+) and (-) foci induced slower growing tumours.

Correlation of focus properties to tumourigenicity

Table 5.6.1 shows the actual data for focus cell lines examined in all studies. The table presents the tumour incidence, focus frequencies on confluent and mixed monolayers as well as the ratios of the growth parameters and cytogenetic data of the foci relative to the untransformed C3H10T½ cells. The ratios of the growth parameters were first calculated as the ratio of parameters in standard to reduced growth conditions and these calculated ratios then compared to the untransformed cells. Data for each focus property were arranged in ascending numerical order and assigned a corresponding rank of one to eighteen (eighteen cell lines examined) and these ranks are presented in table 5.6.2. Wilcoxon's rank sum test was used to assess if significant

differences occurred between tumourigenic and non-tumourigenic cell lines for any of the focus properties examined. A significant difference was found between the tumourigenic and non-tumourigenic cell lines for the focus frequencies on the mixed monolayers only, with an increased frequency occurring for the tumourigenic cell lines ($0.01 < p < 0.05$). Two cell lines (X9, X11) produced only one tumour and were included in the tumourigenic group of cell lines in the above assessment. When the Wilcoxon rank sum test was repeated on the basis of a low (one or no tumours) versus high (greater than one tumour produced) tumourigenicity a significant difference was found between the groups for the focus frequencies on both the mixed and confluent monolayers ($p < 0.01$).

A rank correlation test was carried out to examine correlation between the focus frequency on the mixed monolayers and the other focus properties (other than tumourigenicity) for all eighteen cell lines. A strong correlation was observed between the focus frequencies on the mixed and confluent monolayers as expected, while a negative correlation was noted between the focus frequency on the mixed monolayers and both the mean and modal chromosome number ratios ($0.01 < p < 0.05$). The correlation between the focus frequency and the cytogenetics data was marginally stronger when only tumourigenic foci were examined. Cytogenetics data were not correlated with any of the other focus properties using the same statistical analysis.

Examination of the ranks assigned to the non-tumourigenic foci presented in table 5.6.2. shows that the doubling time and saturation density ratios contain the extremes of the ranks (1 to 6, 15 to 18) with little representation of the middle of the rank range (7 to 14). These properties were rearranged, this time in increasing order of the absolute difference between each ratio and the mean value of the cell lines. The Wilcoxon rank sum test in this case revealed significant differences between the tumourigenic and non-tumourigenic cell lines for both the doubling time and saturation density ratios ($p < 0.01$). The ratios of the doubling time and saturation density were first calculated as the ratio of each parameter in standard to reduced growth conditions and these calculated ratios then compared to the untransformed cells. Thus the highest and lowest ratios of both doubling time and saturation density appear correlated to the absence of tumourigenicity. The differences in the ratios of doubling time and

saturation density were considerably less significant when lower versus higher tumourigenicity was assessed as described above.

There are limitations in the analysis of these data, many of which have already been highlighted in the previous sections of this chapter. The limitations include the small sample population and the considerable uncertainties associated with some of the data points. However a number of trends appear such as the correlation between tumourigenicity and increased focus frequency (on mixed and confluent monolayers) and the negative correlation between the focus frequency (on mixed and confluent monolayers) and the cytogenetics data. Also it would appear that extremes of both saturation density and doubling time ratios are negatively correlated with tumourigenicity. These trends highlight the complex nature of transformation and tumourigenicity. Both transformation and tumourigenicity are complex processes consisting of interactions and relationships between several parameters and one cannot in this case precisely define what is cause and what is effect, for example, the tumourigenicity may be correlated to focus frequency because of the correlation between focus frequency and the cytogenetics data although the data may not be sufficient to show a direct correlation between tumourigenicity and cytogenetics.

Table 5.6.1. Data of focus properties.

Focus			Focus frequencies ($x10^{-4}$)		Ratio of growth *in vitro* parameters			Ratio of Cytogenetics data	
Label	Type	T.I.	Confluent	Mixed	Lag time	D.T	Saturation density	Mean	Modal
CTL		0/30	< 0.4	< 0.4	1.0	1.0	1.0	1.0	1.0
X1	(+)	4/4	207.8 ± 28.8	79 ± 17.8	1.37	1.71	1.17	0.92	0.88
X9	(+)	1/7	2.8 ± 2.8	< 2.8	1.59	1.57	1.07	0.99	0.95
X19	(+)	5/5	260.5 ± 25.8	260.5 ± 25.8	0.86	1.78	1.22	0.91	0.82
α1	(+)	0/4	< 5.4	< 5.4	1.38	1.19	0.81	1.2	0.92
α10	(+)	6/6	113.3 ± 20.8	81.7 ± 17.6	1.01	1.49	1.02	0.97	0.96
α12	(+)	0/4	113.5 ± 20.8	31.7 ± 11	1.0	1.89	1.3	1.01	0.94
α19	(+)	4/4	100.5 ± 18.4	28 ± 9.7	1.54	1.29	0.88	0.95	0.91
STR	(+)	5/5	48.9 ± 13.5	38.9 ± 12.0	0.97	1.11	1.12	1.22	0.95
X4	(X/+)	4/5	18.1 ± 6.1	12 ± 5	1.06	1.56	1.07	1.01	0.97
X12	(X/+)	0/4	< 4.6	4.5 ± 4.4	0.92	2.22	1.52	0.93	0.92
α5	(X/+)	4/4	86.2 ± 15.8	72.2 ± 15.0	0.91	1.38	0.94	0.99	0.81
α13	(X/+)	0/4	33.7 ± 11.7	29.9 ± 11.6	1.18	2.14	1.46	0.91	0.88
X2	(X)	3/3	112.3 ± 19.5	112.3 ± 19.5	1.04	1.53	1.05	0.97	0.94
X11	(X)	1/6	9.4 ± 5.2	12.9 ± 6.1	1.07	1.44	0.99	1.01	0.95
X6	(-?)	0/4	0.4 ± 0.4	< 0.4	1.10	1.37	0.94	0.95	0.9
X14	(-?)	6/10	16.4 ± 8.8	23.1 ± 10.5	0.8	1.59	1.09	1.34	1.39
X18	(-)	9/9	559 ± 193.8	364.5 ± 163.5	0.73	1.98	1.35	0.88	0.84

Table shows the tumour incidence (T.I.), focus frequencies, and the ratios of growth parameters and cytogenetics data relative to the untransformed cells (CTL). Tumour incidence is presented as the number of mice with tumours of the total number of mice injected. The focus frequencies are the frequencies of reconstructed foci (includes all foci irrespective of focus category) per viable focus cell produced by the foci seeded on confluent and mixed monolayers. The ratios of the lag times, doubling times (D.T.) and saturation densities were first calculated as the ratio of parameters in standard to reduced growth conditions and these calculated ratios then compared to the untransformed cells. Mean and modal chromosome numbers are also relative to the untransformed cells (CTL). X-ray induced foci are denoted with an X, alpha-particle induced foci are denoted with α and STR refers to the spontaneous focus.

Table 5.6.2. Focus properties arranged in ascending numerical order.

Focus			Rank of focus frequencies		Rank of ratio of growth *in vitro* parameters			Rank of ratio of cytogenetics data	
Label	Type	T.I.	C	M	Lag time	D.T	Saturation density	Mean	Modal
CTL		0/30	1	1	7.5	1	6	12	17
α1	(+)	0/4	5	5	16	3	1	16	8.5
α12	(+)	0/4	15	11	7.5	15	15	14	10.5
X12	(X/+)	0/4	4	4	5	18	18	5	8.5
α13	(X/+)	0/4	9	10	14	17	17	2.5	4.5
X6	(-?)	0/4	2	2	13	5	3.5	6.5	6
X9	(+)	1/7	3	3	18	11	9.5	10.5	13
X11	(X)	1/6	6	7	12	7	5	14	13
X1	(+)	4/4	16	14	15	13	13	4	4.5
X19	(+)	5/5	17	17	3	14	14	2.5	2
α10	(+)	6/6	14	15	9	8	7	8.5	15
α19	(+)	4/4	12	9	17	4	2	6.5	7
STR	(+)	5/5	10	12	6	2	12	17	13
X4	(X/+)	4/5	8	6	11	10	9.5	14	16
α5	(X/+)	4/4	11	13	4	6	3.5	10.5	1
X2	(X)	3/3	13	16	10	9	8	8.5	10.5
X14	(-?)	6/10	7	8	2	12	11	18	18
X18	(-)	9/9	18	18	1	16	16	1	3

Table shows the focus properties, tabulated in table 5.6.1., arranged according to non-tumourigenic and tumourigenic foci with the corresponding rank assigned in ascending numerical order for each focus property. X-ray induced foci are denoted with an X, alpha-particle induced foci are denoted with α and STR refers to the spontaneous focus.

Conclusions

The studies presented in this thesis show that the induction of transformation is proportional to dose, at least in the range of 0.25 to 5 Gy X-rays and the transformation frequency can be reduced by lowering the dose-rate. Further analysis of the properties of transformed cell phenotypes revealed a positive correlation between tumourigenicity and the ability of transformed cells to reconstruct foci *in vitro* and furthermore the ability to reconstruct foci *in vitro* is negatively correlated to the mean and modal chromosome numbers.

Transformation is a complex multistage process *in vitro* that mimics certain aspects of carcinogenesis *in vivo*. The term transformation may have different meanings in biology and in the context of this thesis it refers to the process by which pre-neoplastic cells acquire characteristics of neoplastic cells. In the C3H10T½ mouse cell line transformed cells are identified *in vitro* by the multilayers of cells (termed foci) which appear on a continuous monolayer of untransformed cells. These foci of transformed cells appear as different phenotypes. It is very important to define the important criteria in the transformation assay, for example, the criteria adopted to distinguish positively transformed foci from the rest of the foci observed as well as optimising and standardising the technical details of how the assay is set-up and maintained. This is especially important to allow comparison of data between laboratories. Another important aspect of this assay is to identify and clarify the true parameter of interest, for example, one of the most important aspects of the foci identified in the C3H10T½ transformation assay is their tumourigenic potential, that is, how the observation *in vitro* relates to the *in vivo* situation. While it is not feasible to examine all foci for tumourigenicity it is imperative that individual laboratories using the C3H10T½ assay examine the tumourigenicity of some foci, deemed to be positively transformed, in order to optimise the criteria within the laboratory for the assessment of truly transformed foci. As observed in this thesis the relationship of the transformed foci to tumourigenicity varies between radiation types and this factor also needs to be considered when optimising the transformation assay.

Possible mechanisms by which transformation occurs is that it is the loss of DNA induced by the radiation that triggers the subsequent manifestation of the different transformation phenotypes. It is proposed that this DNA loss results in the loss of a gene(s) responsible for the maintenance of the integrity of the genome and

/ or the loss of regulatory genes and / or activation of genes which allow mutations to occur at higher frequencies. However several authors reported no structural changes in a variety of genes examined in transformed C3H10T½ cells (studies discussed in section 5.5.) and it is proposed that the most likely scenario is that the co-ordinated expression of different genes (overexpression of proto-oncogenes and / or underexpression of tumour suppressor genes) is responsible for the transformation phenotypes observed and this co-ordinated expression could be caused by epigenetic mechanisms induced by genomic instability arising after irradiation of the untransformed C3H10T½ cells. Different combinations of expression of different genes may result in different transformed phenotypes and although the total changes in gene expression may be similar for different transformed phenotypes the sequence of events may be different. It is hypothesised that the different transformed phenotypes observed (originally categorised as I, II and III, categorised as (+), (X/+), (X) and (-) in this thesis) represent different pathways to transformation which are interlinked. This conclusion is mainly drawn from the focus reconstruction studies based on the preferential patterns of reconstructed foci produced by the foci and more especially the tumour cells. It is proposed that the different phenotypes represent different combinations or different sequences of cytogenetic and / or epigenetic changes. This has been found in the studies of the multistage process of carcinogenesis in colorectal cancer where the accumulation of changes is important, not necessarily the order of these changes to produce malignancy (Fearon and Vogelstein 1990).

It is proposed that genomic instability is induced by both high- and low- LET radiation and is long - lived after radiation exposure remaining evident in the tumour cells produced by the foci even after several weeks in subculture manifesting itself in the gain and later loss of some transformation associated properties. The instability may take the form of a genome - wide process affecting DNA replication repair by altering repair genes or their regulation or may be due to alterations of specific genes which monitor the integrity of the genome and ensure that events such as cell cycle and DNA repair proceed accurately.

Genomic instability may manifest itself in the gain and later loss of some transformation phenotypes. The ability to reconstruct foci was greater for alpha - particle induced foci than for the corresponding X-ray induced foci while the reverse was true for the tumour cells. Several studies report instability induced by high- but

not low - LET radiation (Aghamohammadi *et al.* 1988, Kadhim *et al.* 1995). It is proposed that heritable genomic instability can be induced by both X-rays and alpha - particles although to a greater extent by the alpha - particles where the foci are transformed and show the ability to reconstruct foci yet are less tumourigenic than the X-ray induced equivalent and this ability to reconstruct foci is not a stable parameter carried through in tumour cells to the same extent as in the X-ray treated cells. High-LET radiation damage is considered to be qualitatively different to that of low-LET radiation and this is probably due to the different patterns of energy depositions of the radiations as they pass through the cells. High - LET radiation such as alpha - particles deposit their energy in more localised areas than low- LET radiation such as X-rays. Although comparative amounts of DNA damage may be induced cells are better able to repair the damage if the lesions are sufficiently far apart to prevent interaction of damaged sites and this is more likely following low- LET radiation (Brenner 1990, Ward 1994). The interaction of lesions induced by alpha - particles may be stable enough to be carried through a number of cell generations and yet unstable enough to allow further interactions of lesions several cell generations after the radiation exposure.

The data presented in this thesis suggest a number of areas worthy of further investigation. Firstly the quantitative analysis of the transformation frequencies, especially the effect of dose-rate are warranted to compare these effects at different doses and dose-rates. The qualitative comparison of transformed cells induced by X-rays and alpha-particles presents a number of possibilities for future work. A library of approximately one hundred and thirty cell lines are available to study using molecular biology techniques. These techniques are widely available to examine the DNA damage induced by radiation and can be used to examine the induction of genomic instability by this or related damage. The different categories of foci (types I, II and III or (+), (X/+), (X) and (-)) can be examined for oncogene expression to determine if different patterns of co-ordinated oncogene expression emerge for the different focus categories. Further analyses on the role of DNA repair can be carried out to examine possible differences in the activity of replication enzymes between the different transformation phenotypes induced by high-and low-LET radiation, an area of special interest in genomic instability studies. The advent of techniques such as fluorescence *in situ* hybridisation (FISH) allows the detailed examination of

chromosome exchanges and possible differences in the patterns of exchanges observed in different transformation phenotypes can be ascertained. These suggested studies would provide valuable insight into mechanisms of transformation of C3H10T½ cells by high- and low-LET radiation.

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